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Steven L. Highlander

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gary L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHODS AND COMPOSITIONS FOR THE DIAGNOSIS AND TREATMENT OF CANCER

Group Art Unit: 1632

Examiner: Joseph T. Woitach

Atty. Dkt. No.: INRP:041/SLH

APPEAL BRIEF

MS APPEAL BRIEF - PATENTS
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Appellant hereby submits an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Office Action dated February 23, 2005. A Notice of Appeal was filed on May 23, 2005, and received by the Patent Office on May 25, 2005. The fee for filing this Appeal Brief is $250, and is attached hereto. A request for a one-month extension of time to respond is included herewith along with the required fee. This one-month extension will bring the due date to August 25, 2005, which is within the six-month statutory period.

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PATENT & TRADEMARK OFFICE

I. REAL PARTY IN INTEREST

The real parties in interest are the assignee, The Board of Regents, University of Texas System, and the exclusive licensee, Introgen Therapeutics.

II. RELATED APPEALS AND INTERFERENCES

The present application (Ser. No. 08/758,033) was the subject of Appeal No. 2000-0742. Two Orders Remanding to the Examiner were issued in this appeal. The first Order was issued August 28, 2000, and the second Order was issued March 20, 2002. Copies of both Orders are provided in the Related Proceedings Appendix.

A second Notice of Appeal and Appeal Brief concerning the present application were filed on March 9, 2004. The Examiner issued a further Office Action raising new grounds for rejection on June 3, 2004. This Office Action was superceded by a Supplemental Office Action issued February 23, 2005. It is from this Supplemental Office Action that Appellant presently appeals.

Appellant also filed a Notice of Appeal, which was received by the Patent Office on May 27, 2005, in application Serial No. 09/968,958. Application Serial No. 09/968,958 is a continuation application of the present application.

III. STATUS OF THE CLAIMS

Claims 1-25 were filed with the original application. Claims 26-150 have been added. Claims 15, 21-25, 78, and 79 were canceled in the first response; claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144, and 145 were canceled in an amendment filed concurrent with the first Appeal Brief; and claims 38-68, 73-77, 80-103, 108-132, 137-139, 142, and 143 were canceled in an amendment filed concurrent with the Reply Brief. Claim 10 was canceled in the Preliminary Amendment on Remand filed June 5, 2002. Thus, claims 1-9, 11-14, 16-20, 26-32,

36, 37, and 146-150 remain pending and are appealed. Appellant notes that the Office Action dated February 23, 2005 incorrectly identified claim 10 as pending. As mentioned above, claim 10 was previously canceled. A copy of the pending claims is provided in the Claims Appendix.

IV. STATUS OF AMENDMENTS

No amendments are pending.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention deals with cancer gene therapy. In particular embodiments, the invention provides methods of inhibiting growth of a tumor cell expressing wild-type p53 in a human subject with a solid tumor comprising the steps of: providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and parenterally administering said viral expression construct to said subject, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth. Specification, p. 3, ln. 17-21; p. 8, ln. 17-26.

In other embodiments, the invention provides methods of inducing apoptosis in a tumor cell expressing wild-type p53 in a human subject with a solid tumor comprising the steps of: providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and parenterally administering said viral expression construct to said subject, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth. Specification, p. 3, ln. 17-21; p. 8, ln. 17-26.

In certain aspects of the invention the expression vector is administered to the tumor at least a second time. Specification, p. 34, ln. 17-18. In some embodiments, the invention also provides for treatment of a tumor bed resulting from tumor resection. Specification, p. 4, ln. 1-4. In a particular embodiment, the tumor is resected following at least a second administration, and an additional administration is effected subsequent to the resection. Specification, p. 4, ln. 1-4.

In some embodiments of the invention, the expression vector is administered in a volume of about 3 ml to about 10 ml. Specification, p. 4, ln. 4. In certain embodiments, the amount of adenovirus in each administration is between about 10^7 and 10^{12} pfu. Specification, p. 4, ln. 4-6. In some aspects of the invention, the expression construct is administered by multiple injections comprising 0.1-0.5 ml volumes spaced about 1 cm apart. Specification, p. 34, ln. 17-18. In certain aspects of the invention, the expression construct is administered to the tumor at least six times within a two week treatment regimen. Specification, p. 34, ln. 20-22. In some embodiments the expression vector is administered to a body cavity by continuous perfusion. Specification, p. 33, ln. 18-25. The p53-encoding polynucleotide may be tagged so that expression of p53 from the expression vector can be detected. Specification, p. 3, ln. 26-27. In certain aspects, the tag is a continuous epitope tag. Specification, p. 3, ln. 27-28.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 1-9, 11-14, 16-20, 26-32, 36, 37. and 146-150 stand provisionally rejected for non-statutory double patenting over claims 26-88 of co-pending Application No. 09/968,958.

Claims 1-9, 11-14, 16-20, 26-32, 36, 37, and 146-150 stand rejected under 35 U.S.C. § 103 as being unpatentable over Roth *et al.* (U.S. Patent 6,069,134) (Exhibit A), Liu *et al.* (Exhibit B), Vogelstein *et al.* (U.S. Patent 6,677,312) (Exhibit C), in view of Baker *et al.*

(Science 249(4971):912-915) (Exhibit D) and Shaw *et al.* (PNAS 89:4495-4499) (Exhibit E). Copies of these references are provided in the Evidence Appendix.

VII. ARGUMENT

A. Substantial Evidence is Required to Uphold the Examiner's Position.

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. The Provisional Double Patenting Rejection

Claims 1-9, 11-14, 16-20, 26-32, 36, 37, and 146-150 are provisionally rejected for non-statutory double patenting over claims 26-88 of co-pending Application No. 09/968,958 (the "'958 application"). The Examiner asserts that claim 1 of the present application is essentially the same as claim 58 of the '958 application. The Examiner also asserts that claim 26 of the '958 application encompasses essentially the same invention as encompassed by claims 1 and 12 of the present application. Appellant traverses this rejection.

Appellant submits that claim 1 of the present invention does not encompass the same invention as either claim 26 or 58 of the '958 application. Claim 1 of the present invention is directed to a method of inhibiting growth of a tumor cell expressing ***wild-type*** p53 in a human subject with a solid tumor. Claim 58 of the '958 application is directed to a method of inhibiting growth of a tumor cell in a human subject having a solid tumor comprising a step of ***surgically revealing*** the tumor. Claim 26 of the '958 application is directed to a method for inhibiting microscopic residual tumor cell growth in a human subject comprising steps of identifying a human subject having a resectable tumor and ***resecting*** the tumor. It would not have been obvious to a person of ordinary skill in the art at the time the present invention was made to treat tumor cells having endogenous, wild-type p53 with an expression construct that itself encodes for p53 because, at the time the present invention was made, the conventional thinking in the art was that only tumor cells deficient in p53 could be treated using a p53 expression construct. Thus, claim 1 of the present application is patentably distinct from claims 26 and 58 of the '958 application.

Appellant further notes that a provisional double-patenting rejection is not a final rejection that blocks the prosecution of all of the conflicting applications. If a provisional double-patenting rejection is the only rejection remaining in an application, the Examiner should withdraw the rejection and permit the application to issue as a patent. *Manual of Patent Examining Procedure*, § 804(I)(B), p. 800-15. After one application issues as a patent, the provisional double-patenting rejection in the remaining application is converted to an actual double patenting rejection. *Id.* Once either the present application or the '958 application issues as a patent, Appellant will file a terminal disclaimer, if appropriate, in the remaining pending application.

C. Rejection Under 35 U.S.C. § 103

1. The Legal Standard for Obviousness

Appellant respectfully notes the high standard by which an obviousness argument based on a combination of references is judged. An evaluation of obviousness requires that one consider the invention as a whole. *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1537 (Fed. Cir. 1983). Furthermore, all of the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 985 (C.C.P.A. 1974). In addition, in the case of *In re Vaeck,* 947 F.2d 488, 493 (Fed. Cir. 1991), the Federal Circuit stated that an Examiner must establish at least two additional criteria in order to make a *prima facie* case of obviousness:

1) the prior art would have suggested to one of ordinary skill in the art to make the composition as claimed; and

2) the prior art demonstrates a reasonable expectation of success of the invention.

In re Vaeck also emphasized that both the suggestion and reasonable expectation of success must be found in the prior art, not in the Appellant's disclosure. *Id.*

When an obviousness determination is based on multiple prior art references, there must be a showing of some "teaching, suggestion, or reason" to combine the references. *Gambro Lundia AB v. Baxter Healthcare Corp.*, 110 F.3d 1573, 1579 (Fed. Cir. 1997) (also noting that the "absence of such a suggestion to combine is dispositive in an obviousness determination").

Furthermore, the burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. *See Manual of Patent Examining Procedure* § 2144.03; *Graham v. John Deere Co.*, 383 U.S. 1, 18 (1966). If the Examiner fails to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Rijckaert*, 9 F.3d 1531, 1532

(Fed. Cir. 1993). Even if the Examiner properly meets the burden of showing a *prima facie* case of obviousness, the Appellant may still overcome the obviousness rejection through a showing of secondary considerations such as unexpected results, long felt need, failure by others or commercial success. *See Graham,* 383 U.S. at 17-18.

2. The Claimed Invention is Patentable Over the Cited References

Claims 1-9, 11-14, 16-20, 26-32, 36, 37, and 146-150 stand rejected under 35 U.S.C. § 103 as being unpatentable over Roth *et al.* (U.S. Patent 6,069,134), Liu *et al.*, Vogelstein *et al.* (U.S. Patent 6,677,312) in view of Baker *et al.* (Science 249(4971):912-915) and Shaw *et al.* (PNAS 89:4495-4499). Appellant traverses this rejection.

a) Roth et al. Is Not Available Under § 103

The primary reference cited by the Examiner, Roth *et al.* (U.S. Patent 6,069,134), is not available for establishing the obviousness of the present invention. Roth *et al.* qualifies as prior art only under 35 U.S.C. § 102(e). The present application and Roth *et al.* were, at the time the invention was made, subject to an obligation of assignment to the Board of Regents of the University of Texas System. Thus, pursuant to 35 U.S.C. § 103(c), the subject matter disclosed in Roth *et al.* cannot preclude the patentability of the claimed invention under § 103.

b) The Cited References Do Not Teach All of the Elements of the Claimed Invention

To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 985 (C.C.P.A. 1973). The claimed invention is directed to a method of inhibiting growth of a tumor cell expressing ***wild-type*** p53. As defined in the specification, "wild-type p53" refers to a p53 gene expressing

normal tumor suppressor activity (p. 8, ln. 4-6). The Examiner failed to establish that the cited references teach or suggest this limitation of the claims.

The Examiner alleges that Baker *et al.* and Shaw *et al.* demonstrate that the cell cycle inhibitory affect of p53 can be accomplished by overexpression of p53 even in the presence of endogenous wild-type p53. Specifically, the Examiner alleges that Baker *et al.* teach the delivery to and effect of wild-type p53 on cells that express an endogenous p53, and that Baker *et al.* concluded that wild-type p53 had a suppressive effect on growth. Appellant submits that the Examiner has misinterpreted the teachings of Baker *et al.*, and thus has failed to show where the prior art teaches or suggests the treatment of tumor cells expressing wild-type p53.

Baker *et al.* describe four cell lines: SW837, SW480, RKO, and VACO 235. SW837 and SW480 each have lost one copy of chromosome 17p (including the p53 gene), and the remaining p53 allele in each cell line is mutated (Baker *et al.*, p. 912, col. 3). No mutation in the p53 coding sequence was detected in the RKO cell line in the 5 exons examined; however, the cells expressed low concentrations of p53 mRNA compared to normal cells and did not produce detectable amounts of protein (Baker *et al.*, p. 913, col. 3). Only the VACO 235 cell line was reported to express wild-type p53 mRNA at levels similar to those found in normal cells (Baker *et al.*, p. 914, col. 2). In regard to the VACO 235 cell line, Baker *et al.* stated that ***"transfection with the wild-type gene had no apparent effect on the growth of epithelial cells derived from a benign colorectal tumor that had only wild-type alleles."*** (Baker *et al.*, Abstract; *see also*, p. 914, col. 2).

The Examiner cites Shaw *et al.* as demonstrating that the overexpression of wild-type p53 causes cell cycle arrest at the G1-S boundary and may result in apoptosis. The Examiner,

however, fails to show where Shaw *et al.* teach or suggest that overexpression of wild-type p53 causes cell cycle arrest and apoptosis in tumor cells expressing ***wild-type*** p53. The experiments reported in Shaw *et al.* utilize the human colon tumor cell line EB (Shaw *et al.*, p. 4495, col. 2), which does not express endogenous wild-type p53 (Shaw *et al.*, p. 4498, col. 2). The Examiner also failed to show that Roth, Liu, or Vogelstein teach or suggest a method of inhibiting the growth of a tumor cell expressing wild-type p53.

The Examiner has failed to show where the prior art teaches or suggests all of the limitations of the claimed invention. Accordingly, the Examiner has not made a *prima facie* case that the claimed invention is obvious.

c) The Cited References Teach Away From the Claimed Invention

Not only did the Examiner fail to establish that the cited references teach or suggest all of the limitations of the claimed invention, the cited references actually teach away from the claimed invention. A "reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Appellant." *Tec Air Inc. v. Denso Mfg. Michigan Inc.*, 192 F.3d 1353, 1360 (Fed. Cir. 1999) (citing with approval, *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994)). A prior art reference that "teaches away" from the claimed invention is a significant factor to be considered in determining obviousness. *In re Gurley*, 27 F.3d 551, 554 (Fed. Cir. 1994).

As mentioned above, Baker *et al.* stated that "transfection with the wild-type gene had no apparent effect on the growth of epithelial cells derived from a benign colorectal tumor that had only wild-type alleles." Baker *et al.*, Abstract. Shaw *et al.* disclose studies in a cell line

deficient in endogenous p53. In regard to their observations with this cell line, Shaw *et al.* stated:

> Whether [deficient endogenous p53 expression] is of significance to induction of apoptosis by wt p53 is not known. wt p53 has been expressed in a variety of cell types, including colon tumor cell lines [citing Baker *et al.*]; yet only this report and that of Oren and co-workers [who also used a cell line deficient in endogenous p53] describe induction of apoptosis.

(Shaw *et al.*, p. 4498, col. 2). These statements by Baker *et al.* and Shaw *et al.* would certainly discourage a person of ordinary skill in the art from the path that was taken by the Appellant.

Vogelstein *et al.* also teach away from the use of p53 gene therapy in tumor cells expressing a functional p53. The Vogelstein patent is entitled "Methods For ***Restoring*** Wild-Type p53 Gene Function," and the claims are directed to "[a] method of supplying wild-type p53 gene function ***to a cell which has lost said gene function*** by virtue of a mutation in a p53 gene, comprising:..." (emphasis added). Thus, Vogelstein *et al.* further reinforces that the conventional thinking at the time of Appellant's invention was that p53 gene therapy would only be beneficial in tumors lacking a functional p53 molecule. A person of ordinary skill, upon reading the Vogelstein reference, would be led in a direction divergent from the path that was taken by the Appellant.

Similarly, upon reading the Liu reference, a person of ordinary skill would be led in a direction divergent from the path that was taken by the Appellant. The experiments described in Liu use the SCCHN cell lines Tu-138 and Tu-177, both of which possess a ***mutated*** p53 gene (Liu *et al.*, p. 3663, col. 2). In other words, Liu uses the Ad-p53 construct to compensate for the absence of a functional p53 molecule in tumors lacking that molecule. The Examiner has failed

to show where Liu teaches or suggests that a tumor cell having a functional p53 molecule may be treated using an expression construct that, itself, encodes a p53 molecule.

The references cited by the Examiner only reinforce that the conventional thinking at the time of Appellant's invention was that p53 gene therapy would only be beneficial in tumors lacking a functional p53 molecule.

> It is difficult but necessary that the decisionmaker forget what he or she has been taught...about the claimed invention and cast the mind back to the time the invention was made (often as here many years), to occupy the mind of one skilled in the art who is presented only with the references, and who is normally guided by the then-accepted wisdom in the art.

W.L. Gore & Associates, Inc. v. Garlock, Inc., 721 F.2d 1540, 1553 (Fed Cir. 1983), *cert. denied*, 469 U.S. 851 (1984). At the time the present invention was made, a person of ordinary skill, upon reading the references cited by the Examiner, would have been led in a direction divergent from the path that was taken by Appellant. The Examiner failed to establish a *prima facie* case for obviousness, because the Examiner applied impermissible hindsight in alleging that the claimed invention is unpatentable under 35 U.S.C. § 103.

d) The Cited References Do Not Provide A Reasonable Expectation of Success

To establish a *prima facie* case of obviousness, the prior art must provide a reasonable expectation of success in achieving the claimed invention. The Examiner has the burden to show that the *in vitro* and animal studies are reasonably predictive of human clinical studies. The Examiner has not made this case. The limitations of *in vitro* studies are manifest, and so well supported in the art that it is unnecessary to recount them here. In fact, the first Office Action addressed limitations on gene therapy, and specifically discussed the shortcomings of delivery and expression of transgenes *in vivo*:

> The unpredictability of gene therapy and vector targeting is supported by the teachings of Culver *et al.*, Hodgson *et al.* and Miller *et al.* Culver *et al.*, reviewing gene therapy for cancer, conclude that the "primary factor hampering the widespread application of gene therapy to human disease is the lack of an efficient method for delivering genes *in situ*, and developing strategies to deliver genes to a sufficient number of tumor cells to induce complete tumor regression or restore genetic health remains a challenge" (page 178). Hodgson discusses the drawbacks of viral transduction and chemical transfection methods, and states that "[d]eveloping the techniques used in animal models, for therapeutic use in somatic cells, has not been straightforward" (pages 459-460). Miller *et al.* also review the types of vectors available for *in vivo* gene therapy, and conclude that "for all the long-term success as well as the widespread applicability of human gene therapy, there will have to be advances ... targeting strategies outlined in this review, which are currently only at the experimental level, will have to be translated into components of safe and highly efficient delivery systems" (page 198, column 1).

First Office Action, pages 5-6.

The art is replete with examples of cancer treatments that showed promise *in vitro* only to fail *in vivo*. For example, Planchon *et al.* (1992) (Exhibit F) showed that butyrate derivatives inhibited growth of breast cancer cell monolayers *in vitro*, but failed to affect the rate of tumor growth *in vivo*. Welters *et al.* (1999) (Exhibit G), in examining the effects on cisplatin in head & neck cancers, found a lack of correlation between studies on *in vitro* tumor cell lines and *in vivo* tumors. Vingerhoeds *et al.* (1996) (Exhibit H) similarly compared the effects of doxorubicin on ovarian carcinoma cells and found that *in vitro* inhibition was not observed *in vivo*. Mourad *et al.* (1996) (Exhibit I) showed that high doses of vitamin A inhibited head & neck and lung cancers *in vitro*, but showed no similar effects *in vivo*. Liu *et al.* (2000) (Exhibit J) disclosed that, *in vivo*, secretion of TGF-β correlated with resistance to tumor therapy, while no correlation was observed *in vitro*. Finally, Johansson *et al.* (1991) (Exhibit K) demonstrated that a murine monoclonal antibody inhibited cancer cells *in vitro*, but that *in vivo* inhibition was limited to two days after inoculation into animals, hardly a clinically relevant situation.

Furthermore, animal models of various human diseases are not necessarily a reliable indicator of the therapeutic benefit of treatments in humans as cited in Crystal (*Science*, 270, 1995, 404-410) (Exhibit L), Gomez-Navarro *et al.* (*European J. of Cancer*, 35(6), 1999, 867-885) (Exhibit M), and Sigmund (*Arterioscler. Thromb. Vasc. Biol.*, 20, 2000, 1425-1429) (Exhibit N). Crystal notes that "[t]here have been several surprise examples, in which predictions from gene transfer studies in experimental animals have not been borne out in human safety and efficacy trials" (p. 409). This sentiment is echoed in Gomez-Navarro *et al.*, which states that the successful treatments found in animal models are not consistently found in humans (p. 875). Additionally, Sigmund expresses concern for the various findings within the same species, only on different genetic backgrounds (p. 1425).

The references cited by the Examiner do not refer to any human clinical findings, only to findings *in vitro* and in animal model systems (specifically the nude mouse model). In contrast, Appellant has provided scientific proof of human clinical data on the benefits of the wild-type p53 viral vector expression system in suppressing growth of HNSCC in tumors having either wild-type p53 or mutant p53. Moreover, as discussed in the preceding section, the references cited by the Examiner actually expressed doubt regarding the benefit of using p53 gene therapy in tumor cells expressing wild-type p53. Thus, the prior art could not have provided a reasonable expectation of success at the time the present invention was made.

e) Conclusion

Appellant has provided numerous reasons as to why claims 1-9, 11-14, 16-20, 26-32, 36-37, and 146-150 are not obvious in view of Roth *et al.*, Liu *et al.*, Vogelstein *et al.*, Baker *et al.*, and Shaw *et al.*. First, the primary reference, Roth *et al.*, is not available as prior art for a rejection under § 103. Second, the Examiner failed to establish a *prima facie* case for

obviousness, because the Examiner failed to show where the cited art teaches or suggests all of the limitations of the claimed invention. Third, the cited art actually teaches away from the treatment of tumor cells expressing wild-type p53 with vectors expressing p53. Finally, those of skill in the art would not have had a reasonable expectation of success based on the cited art.

For the reasons described above, claims 1-9, 11-14, 16-20, 26-32, 36-37, and 146-150 are not obvious in view of Roth *et al.*, Liu *et al.*, Vogelstein *et al.*, Baker *et al.*, and Shaw *et al.*. Appellant requests that the Board overturn this rejection.

3. Additional Arguments for the Separate Patentability of Specific Claims

The arguments set forth above are directed to the non-obviousness of all of the pending claims. Appellant will now present additional arguments for the separate patentability of various dependent claims, which do not stand or fall together.

a) Claim 12 and 18

Claim 12 contains the limitation of the resection of a tumor cell expressing wild-type p53 in a human subject following at least a second administration of the expression vector, and an additional administration of the expression vector is effected subsequent to the resection. Claim 18 contains the limitation of "an artificial body cavity resulting from tumor excision." The Examiner failed to show where the references teach or suggest tumor resection and the treatment of the tumor bed resulting from tumor resection/excision.

As described in the present specification, one of the shortcomings of conventional cancer treatments is the inability to completely eradicate disease (*i.e.,* microscopic residual disease) at the primary tumor site (p. 35, ln. 7-11). In certain embodiments, the present invention is designed to provide methodologies that permit a more complete and effective treatment of cancer

by attacking microscopic residual disease. Methods for using the expression vectors of the claimed invention in the treatment of artificial body cavities created by tumor resection are described in the present specification at, for example, p. 34, ln. 24 to p. 35, ln. 4; and p. 35, ln. 7-25).

The Examiner failed to show where the references teach or suggest the additional limitations recited in claims 12 and 18 and, therefore, failed to establish a *prima facie* case of obviousness for these claims.

b) Claim 13

Claim 13 recites the limitation of administering the expression vector "in a volume of about 3 ml. to about 10 ml." The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 13 is obvious in view of the prior art. The Examiner notes that Liu *et al.* administered 100 μl volumes (Action, p. 8). Apart from that disclosure, the Examiner merely states that Roth *et al.* teach to optimize delivery for a specific vector for different PFU and volumes, and that Vogelstein *et al.* teach that delivery can be tailored to the type of cancer being treated.

Again, as mentioned above, Roth *et al.* is not available as prior art for an obviousness rejection. With regard to Vogelstein *et al.*, Appellants could locate no description of any specific volumes for administration, much less of a volume of about 3 ml to about 10 ml. The Examiner's conclusory statements fail to establish that the specific volumes recited in claim 13 would have been obvious to a person of ordinary skill in the art at the time the present application was filed.

c) Claim 17

Claim 17 contains the limitation of "continuous perfusion" of a natural or artificial body cavity with a viral expression construct encoding a functional p53 polypeptide for inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 17 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest "continuous perfusion." Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

d) Claim 18

Claim 18 recites the limitation of administering the expression construct into "an artificial body cavity resulting from tumor excision." The Examiner asserts that Roth *et al.* disclose delivery to a tumor bed from which a tumor has been removed. However, Roth *et al.* is not available as prior art for an obviousness rejection. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has presented no evidence to support the rejection of claim 18; thus, the Examiner has failed to establish a *prima facie* case for the obviousness of claim 18.

e) Claims 19 and 20

Claims 19 and 20 contain the limitation of a p53-encoding polynucleotide that is tagged so that expression of p53 from said expression vector can be detected. As described in the present specification, certain aspects of the invention involve the monitoring of p53 expression

following administration of the expression construct (p. 36, ln. 14-15). The tagging of the p53-encoding polynucleotide is advantageous in, for example, monitoring the treatment of microscopic residual disease where it is difficult to observe the destruction of microscopic tumor cells (p. 36, ln. 15-17).

The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claims 19 and 20 are obvious in view of the prior art. Therefore, a *prima facie* case for obviousness has not been established.

f) Claim 26

Claim 26 recites the limitation of administering the expression construct to the tumor "at least twice." The Examiner asserts that Roth *et al.* teach that for effective treatment multiple times and multiple sites of delivery may be necessary. However, Roth *et al.* is not available as prior art for an obviousness rejection. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has presented no evidence to support the rejection of claim 26; thus, the Examiner has failed to establish a *prima facie* case for the obviousness of claim 26.

g) Claim 27

Claim 27 recites the limitation of administering multiple injections that "comprise about 0.1-0.5 ml volumes spaced about 1 cm apart." The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 27 is obvious. The Examiner merely states that Roth *et al.* teach that for effective treatment multiple times and multiples sites of delivery may be necessary. Such a conclusory

statement fails to establish a *prima facie* case for the obviousness of the specific volumes and injection spacing recited in claim 27. Moreover, as mentioned above, Roth *et al.* is not available as prior art for an obviousness rejection. The Examiner has presented no evidence to support the rejection of claim 27; thus, the Examiner has failed to establish a *prima facie* case for the obviousness of claim 27.

h) ***Claims 28-32***

Claims 28-32 are directed generally to combining p53 gene therapy with an additional DNA damaging agent. The only evidence presented by the Examiner regarding the suggestion of such methods is Roth *et al.* As mentioned above, Roth *et al.* is not available as prior art for an obviousness rejection. Thus, the Examiner has presented no evidence to support the rejection of claims 28-32. The Examiner, therefore, has not met his burden of establishing a *prima facie* case for obviousness.

i) Claim 37

Claim 37 is directed to a method of administering the expression construct to the tumor "at least six times within a two week treatment regimen." The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 37 is obvious. The Examiner merely states that Roth *et al.* teach that for effective treatment multiple times and multiple sites of administration may be necessary, and that Vogelstein *et al.* teach that delivery can be tailored to the type of cancer being treated. The Examiner has not identified any disclosure that would teach or suggest the claimed treatment regimen. Moreover, Roth *et al.* is not available as prior art for an obviousness rejection. Accordingly, the Examiner failed to establish a *prima facie* case for obviousness.

j) Claim 147

Claim 147 is directed to the intravenous administration of the expression construct. The Examiner has identified no disclosure that teaches or suggest such a route of administration. The Examiner notes that Liu *et al.* disclose delivering the vector to a revealed tumor. The Examiner asserts that Vogelstein *et al.* teaches that the delivery can be tailored to the type of cancer. Appellants, however, could find no disclosure regarding any particular route of administering the expression construct to a subject, much less administering it intravenously. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has not met this burden.

k) Claim 149

Claim 149 is directed to the intraperitoneal administration of the expression construct. The Examiner has identified no disclosure that teaches or suggest such a route of administration. The Examiner notes that Liu *et al.* disclose delivering the vector to a revealed tumor. The Examiner asserts that Vogelstein *et al.* teaches that the delivery can be tailored to the type of cancer. Appellants, however, could find no disclosure regarding any particular route of administering the expression construct to a subject, much less administering it intraperitoneally. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has not met this burden.

VIII. CONCLUSION

It is respectfully submitted, in light of the above, that all of the pending claims are in condition for allowance. Appellant, therefore, requests that the Board overturn each of the pending grounds for rejection.

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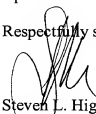
Respectfully submitted,



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Date: July 26, 2005

CLAIMS APPENDIX

1. A method of inhibiting growth of a tumor cell expressing wild-type p53 in a human subject with a solid tumor comprising the steps of:

   (a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and

   (b) parenterally administering said viral expression construct to said subject, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth.

2. The method of claim 1 or 146, wherein said tumor is selected from the group consisting of a carcinoma, a glioma, a sarcoma, and a melanoma.

3. The method of claim 1 or 146, wherein said tumor cell is malignant.

4. The method of claim 1 or 146, wherein said tumor cell is benign.

5. The method of claim 1 or 146, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

6. The method of claim 1 or 146, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

7. The method of claim 6, wherein said viral vector is a replication-deficient adenoviral vector.

8. The method of claim 7, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

9. The method of claim 8, wherein said promoter is a CMV IE promoter.

11. The method of claim 7, wherein the expression vector is administered to said tumor at least a second time.

12. The method of claim 11, wherein said tumor is resected following at least a second administration, and an additional administration is effected subsequent to said resection.

13. The method of claim 1, wherein said expression vector is administered in a volume of about 3 ml. to about 10 ml.

14. The method of claim 11, wherein the amount of adenovirus in each administration is between about 10^7 and 10^{12} pfu.

16. The method of claim 1 or 146, wherein the expression construct is injected into a natural or artificial body cavity.

17. The method of claim 16, wherein said injection comprises continuous perfusion of said natural or artificial body cavity.

18. The method of claim 16, wherein said body cavity is an artificial body cavity resulting from tumor excision.

19. The method of claim 1 or 146, wherein the p53-encoding polynucleotide is tagged so that expression of p53 from said expression vector can be detected.

20. The method of claim 19, wherein the tag is a continuous epitope.

26. The method of claim 1 or 146, wherein said expression construct is administered to said tumor at least twice.

27. The method of claim 26, wherein said multiple injections comprise about 0.1-0.5 ml volumes spaced about 1 cm apart.

28. The method of claim 1 or 146, further comprising contacting said tumor with a DNA damaging agent.

29. The method of claim 28, wherein said DNA damaging agent is a radiotherapeutic agent.

30. The method of claim 29, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

31. The method of claim 28, wherein said DNA damaging agent is a chemotherapeutic agent.

32. The method of claim 31, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

36. The method of claim 1 or 146, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

37. The method of claim 11, wherein said expression construct is administered to said tumor at least six times within a two week treatment regimen.

146. A method of inducing apoptosis in a tumor cell expressing wild-type p53 in a human subject with a solid tumor comprising the steps of:

(a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and

(b) parenterally administering said viral expression construct to said subject, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth.

147. The method of claim 1 or 146, wherein the expression construct is administered intravenously.

148. The method of claim 1 or 146, wherein the expression construct is administered by direct injection into the tumor.

149. The method of claim 1 or 146, wherein the expression construct is administered intraperitoneally.

150. The method of claim 1 or 146, wherein the expression construct is administered orthotopically.

[CANCER RESEARCH 54, 3662–3667, July 15, 1994]

Advances in Brief

Growth Suppression of Human Head and Neck Cancer Cells by the Introduction of a Wild-Type *p53* Gene via a Recombinant Adenovirus[1]

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Abstract

Mutations of the *p53* gene constitute one of the most frequent genetic alterations in squamous cell carcinoma of the head and neck (SCCHN). In this study, we introduced wild-type *p53* into two separate SCCHN cell lines via a recombinant adenoviral vector, Ad5CMV-p53. Northern blotting showed that following infection by the wild-type *p53* adenovirus (Ad5CMV-p53), cells produced up to 10-fold higher levels of exogenous *p53* mRNA than cells treated with vector only (without *p53*). Western blotting showed that the increased levels of p53 protein produced in the Ad5CMV-p53-infected cells were a reflection of *p53* mRNA expression. *In vitro* growth assays revealed growth arrest following Ad5CMV-p53 infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral intratumoral infiltration of Ad5CMV-p53. These data suggest that Ad5CMV-p53 may be further developed as a potential novel therapeutic agent for SCCHN since introduction of wild-type p53 into SCCHN cell lines attenuates their replication and tumor growth.

Introduction

Patients with SCCHN[3] are afflicted with a disease process that often has profound effects on speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients has remained unchanged at approximately 45% for nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Treatment failures among these patients remain local and regional, only 10–15% of patients with the disease die of distant metastasis alone (2).

Although we have gained an understanding of the molecular events in the initiation and progression of SCCHN, they continue to require intensive investigation. A recent study identifying loss of heterozygosity of chromosome 9p21–22 as the most frequent genetic alteration in SCCHN suggested that this may be an early event in progression toward this neoplasm (3). Additionally, amplification and/or overexpression of cellular and nuclear oncogenes, such as *c-erB-1* (4), *int-2* (5), *bcl-1* (6) and *c-myc* (7), have been documented in these cancers. The tumor suppressor gene *p53* has been the subject of immense interest and investigation in recent years. Alterations in the *p53* gene, including deletion, insertion, and point mutation, are the most frequent genetic events in many different carcinomas, such as those of the colon (8), breast (9), and lung (10), as well as soft-tissue sarcomas and leukemias (11). Several investigators have demonstrated the high frequency of *p53* gene alterations in SCCHN (12, 13).

There is considerable evidence implicating mutations of the *p53* gene in the etiology of many human cancers (14). Reports have demonstrated that growth of several different human cancer cell lines, including representatives of colon cancer (15), glioblastoma (16), breast cancer (17), and osteosarcoma (18), can be functionally suppressed by DNA transfection or retrovirus-mediated transfer of the wild-type *p53* gene. This gene may have an important role not only in cell growth but in apoptosis (programmed cell death). Induction of exogenous expression of wild-type, *p53* has been shown to induce apoptosis in colon cancer cell lines (19) and in human lung cancer spheroids (20).

The adenoviral vector has emerged as a leading candidate for *in vivo* gene therapy in the past few years. It enjoys an advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells (21). The recently created adenoviral vector containing wild-type *p53* (Ad5CMV-p53; Ref. 22) provides us with an attractive delivery system to investigate the effect of exogenous wild-type *p53* on SCCHN cell lines both *in vitro* and *in vivo*. The outcome of this study indicates that further development of the p53 adenovirus or other novel molecular therapies for SCCHN is warranted.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines Tu-138 and Tu-177 were both established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center. Tu-138 and Tu-177 were established from a gingivo-labial moderately differentiated squamous carcinoma and a poorly differentiated squamous carcinoma of the larynx, respectively. Both cell lines were developed via primary explant technique and are cytokeratin positive and tumorigenic in athymic nude and SCID mice. These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS with penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection. The recombinant p53 adenovirus (Ad5CMV-p53; Ref. 22) contains the CMV promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in phosphate-buffered saline, and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Infection was carried out by the addition of the virus to the DMEM/F12 medium and 2% FBS to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Then complete medium (DMEM/F12–10% FBS) was added, and the cells were incubated at 37°C for the desired length of time.

Northern Blot Analysis. Total RNA was isolated by the acid-guanidinium thiocyanate method of Chomczynski and Sacchi (23). Northern analyses were performed on 20 μg of total RNA. The membrane was hybridized with a *p53*

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[2] To whom requests for reprints should be addressed, at M. D. Anderson Cancer Center, Department of Head and Neck Surgery, Box 69, 1515 Holcombe Boulevard, Houston, TX 77030.

[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; FBS, fetal bovine serum; Ad5, adenovirus 5; CMV, cytomegalovirus; Ad5CMV-p53, wild-type p53 adenovirus; cDNA, complementary DNA; MOI, multiplicity of infection; SDS, sodium dodecyl sulfate; β-gal, β-galactosidase; dl312, replication-defective adenovirus; PFU, plaque forming unit.

cDNA probe labeled by the random primer method in 5 × SSC-5 × Denhardt's solution-0.5% SDS-denatured salmon sperm DNA (20 μg/ml). The membrane was also stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for RNA loading control. The relative quantities of *p53* expressed were determined by densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blot Analysis. Total cell lysates were prepared by sonicating the cells 24-h postinfection in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) for 5 s. Fifty μg of protein from samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham). The membrane was blocked with Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in phosphate-buffered saline) and probed with the primary antibodies, mouse anti-human p53 monoclonal antibody PAb1801 and mouse anti-human β-actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). The membrane was processed and developed as the manufacturer suggested.

Immunohistochemical Analysis. The infected cell monolayers were fixed with 3.8% formalin and treated with 3% H_2O_2 in methanol for 5 min. Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector, Burlingame, CA). The primary antibody used was the anti-p53 antibody PAb1801, and the secondary antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase avidin-biotin complex reagent was used to detect the antigen-antibody complex. Preadsorption controls were used in each immunostaining experiment. The cells were then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

Cell Growth Assay. Cells were plated at a density of 2×10^4 cells/ml in 6-well plates in triplicate. Cells were infected with either wild-type (Ad5CMV-p53) or replication-deficient adenovirus as a control. Cells were harvested every 2 days and counted; their viability was determined by trypan blue exclusion.

Inhibition of Tumor Growth *in Vivo*. The effect of Ad5CMV-p53 on established s.c. tumor nodules was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and use and for recombinant DNA research. Briefly, following induction of acepromazine/ketamine anesthesia, three separate s.c. flaps were elevated on each animal, and 5×10^6 cells in 150 μl of complete media were injected s.c. into each flap using a blunt needle; the cells were kept in the pocket with a horizontal mattress suture. Four animals were used for each cell line. After 4 days, the animals were reanesthetized, and the flaps were reelevated for the delivery of (00 μl of: (*a*) Ad5CMV-p53 (10^8 PFU) in the right anterior flap; (*b*) replication-deficient virus (10^8 PFU) in the right posterior flap; and (*c*) transport medium alone, in the left posterior flank. All injection sites had developed s.c. vitual and palpable nodules before treatment was administered. Animals were observed daily and sacrificed on day 20. *In vivo* tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Following sacrifice, excised tumors were measured three dimensionally by microcalipers to determine tumor volume. A nonparametric Friedman's two-way analysis of variance test was used to test the significance of the difference between means of samples; the SPSS/PC+ software package (SPSS, Inc., Chicago, IL) was used.

Results

Adenoviral Infection of SCCHN Cells. The conditions for optimal adenoviral transduction of Tu-138 and Tu-177 cells were determined by infecting these cells with adenovirus expressing the *Escherichia coli* β-gal gene. The transduction efficiency was assessed by counting the number of blue cells after X-gal staining. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used. Cells inoculated with a single dose of 100 MOI β-gal adenovirus exhibited 60% blue cells (Fig. 1*A*), and this was improved to 100% by multiple infections (data not shown). The transduction efficiency of this vector in SCCHN cells is quite different from that of other cell lines examined previously: HeLa, HepG2, LM2, and human non-small cell lung cancer cell lines

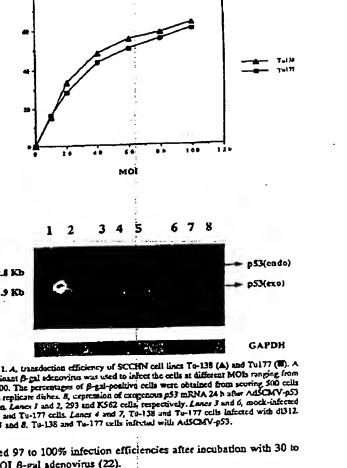


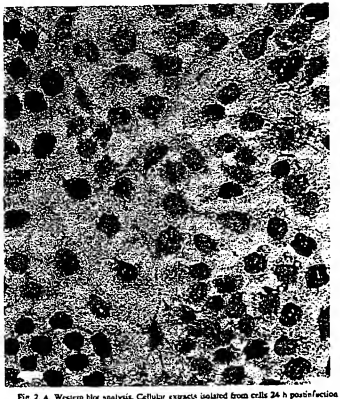
Fig. 1. *A*, transduction efficiency of SCCHN cell lines Tu-138 (▲) and Tu177 (■). A recombinant β-gal adenovirus was used to infect the cells at different MOIs ranging from 10 to 100. The percentages of β-gal-positive cells were obtained from scoring 500 cells each on replicate dishes. *B*, expression of exogenous *p53* mRNA 24 h after Ad5CMV-p53 infection. *Lanes 1* and *2*, 293 and K562 cells, respectively. *Lanes 3* and *6*, mock-infected Tu-138 and Tu-177 cells. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 5* and *8*, Tu-138 and Tu-177 cells infected with Ad5CMV-p53.

showed 97 to 100% infection efficiencies after incubation with 30 to 50 MOI β-gal adenovirus (22).

Expression of Exogenous *p53* mRNA in Adenovirus-infected SCCHN Cells. Two human SCCHN cell lines were chosen for this study; both cell lines Tu-138 and Tu-177 possess a mutated *p53* gene (unpublished data). The recently created recombinant wild-type p53 adenovirus, Ad5CMV-p53, was used to infect Tu-138 and Tu-177 cells. Twenty-four h after infection, total RNA was isolated, and Northern blot analysis was performed. The transformed primary human embryonal kidney cell line 293 was used as a positive control because of its high level of expression of the *p53* gene product, whereas K562, a lymphoblastoma cell line with a homozygous deletion of the *p53* gene, was the negative control (Fig. 1*B*, *Lanes 1* and *2*, respectively). Due to unequal loading, only a fraction of the endogenous *p53* mRNA was detected in the 293 cells (Fig. 1*B*, *bottom panel*). The levels of the 2.8-kilobase endogenous *p53* mRNA detected in the samples isolated from mock-infected cells (Fig. 1*B*, *Lanes 3* and *6*) and from the cells infected with a replication-defective adenovirus, dl312 (Fig. 1*B*, *Lanes 4* and *7*), were similar. Up to 10-fold higher levels of exogenous 1.9-kilobase *p53* mRNA were present in the cells infected with Ad5CMV-p53 (Fig. 1*B*, *Lanes 5* and *8*), indicating that the exogenous *p53* cDNA was successfully transduced into these cells and efficiently transcribed. Interestingly, the level of endogenous *p53* mRNA in these cells was 5-fold higher than in the experimental controls. Northern blots exhibited no evidence of Ad5CMV-p53 (DNA) contamination of RNA.

Expression of p53 Protein in Adenovirus-infected SCCHN Cells. Western blot analysis was performed to compare the levels of *p53* mRNA to the amount of p53 protein produced. A p53 band, recognized by monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples except K562 cells (Fig. 2*A*, *Lane 8*). Cell line 293 showed high levels of p53 protein (Fig. 2*A*, *Lane 1*). Samples isolated from mock-infected Tu-138 and Tu-177 cells exhibited low levels of p53 protein (Fig. 2*A*, *Lanes 2* and *5*). The level of p53 expression remained similar in those cells infected with the dl312 adenovirus (Fig. 2*A*, *Lanes 3* and *6*). The levels of p53 antigen detected in Ad5CMV-p53-infected cells were significantly higher than the levels of the endogenous mutated proteins in both cell lines (Fig. 2*A*, *Lanes 5* and *7*). This result indicates that the exogenous *p53* mRNA produced from cells infected with Ad5CMV-p53 is efficiently translated into immunoreactive p53 protein. Furthermore, immunohistochemical analysis of cells infected with Ad5CMV-p53 revealed the characteristic nuclear staining of p53 protein (Fig. 2*B*, *right panel*), whereas mock-infected cells failed to show similar staining despite the presence of the p53 protein in these cells (Fig. 2*B*, *left panel*). This inability to detect the protein may be attributable to the insensitivity of the assay.

Effect of Exogenous p53 on SCCHN Cell Growth *in Vitro*. Cells infected with control virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 3), whereas growth of the Ad5CMV-



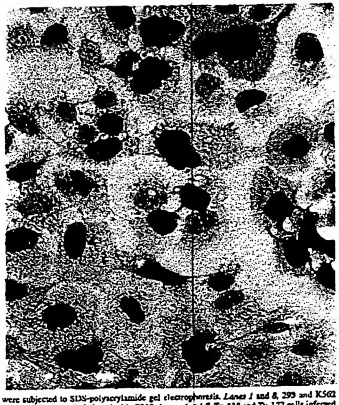


Fig. 2. *A*, Western blot analysis. Cellular extracts isolated from cells 24 h postinfection were subjected to SDS-polyacrylamide gel electrophoresis. *Lanes 1* and *8*, 293 and K562 cells, respectively. *Lanes 2* and *5*, mock-infected Tu-138 and Tu-177 cells. *Lanes 3* and *6*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with the Ad5CMV-p53. *B*, representative immunohistochemical staining of mock-infected Tu-138 cells (*left*) and Ad5CMV-p53-infected Tu-138 cells (*right*) 24-h postinfection. × 250.

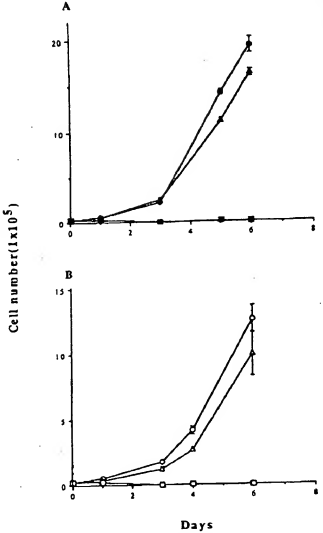


Fig. 3. Inhibition of SCCHN cell growth *in vitro*. *A*, growth curve of mock-infected Tu-138 cells (●), dl312-infected cells (▲), and Ad5CMV-p53-infected cells (■). *B*, growth curve of mock-infected Tu-177 cells (○), dl312-infected cells (△), and Ad5CMV-p53-infected cells (□). At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate wells following infection were plotted against the number of days since infection; *bars*, SEM.

p53-infected Tu-138 (Fig. 3*A*) and Tu-177 (Fig. 3*B*) cells was greatly suppressed. Twenty-four h after infection, an apparent morphological change occurred with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect was more prominent for Tu-138 than for Tu-177 cells. Cells infected with the replication-defective adenovirus, dl312, demonstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

Inhibition of Tumor Growth *in Vivo*. Seven animals were tested for each cell line. One animal in the Tu-177 group died following the second flap surgery and delivery of the therapeutic interventions, presumably due to profound anesthesia and subsequent mutilation by cage mates. Necropsy revealed no evidence of metastasis or systemic effects. Fig. 4 shows representative Tu-138 (*left*) and Tu-177 recipients (*right*). Sizable tumors are apparent on both posterior flaps of the animals (*i.e.*, the sites that did not receive Ad5CMV-p53). The lack of tumor progression is significant in the right anterior flaps of the animals which received Ad5CMV-p53 ($P < .04$). That Tu-177 cells have a slower growth rate has been established previously in these animals.[4] Two animals in the Tu-177 group had complete clinical and pathological regression of their established s.c. tumor nodule. Two animals in the Tu-138 group were killed early because they were experiencing rapid growth and ulceration of the control tumor sites. All surgical sites had developed lesions of at least 6 mm^3 before intervention. The tumor volumes on necropsy are shown in Table 1.

Discussion

Mutations or deletions of the *p53* tumor suppressor gene are the most frequent genetic alterations reported in SCCHN. Since the wild-type *p53* gene is believed to be involved primarily in delivering antiproliferative signals that may be capable of antagonizing the growth-stimulatory signals propagated by oncogene products, the potential molecular therapeutic effect of this gene in SCCHN deserves attention.

The rapid development in the field of gene therapy, including the creation of adenoviral vectors, has created an environment that is well suited for progress toward novel gene therapy of SCCHN. Because of their natural tropism for aerodigestive tract epithelium, adenoviruses may be uniquely suitable for the transient delivery of genes to cancers in these epithelial tissues. The recombinant, replication-defective adenoviruses that have been developed for gene therapy are missing the entire E1a and part of the E1b regions and are, therefore, capable of propagating only in cells that can provide the E1 proteins in trans, such as the 293 cell line. In the past few years, recombinant adenoviruses have been extensively developed and used for *in vivo* gene therapy. The high transfer efficiency of adenoviral vectors over a broad range of hosts both *in vitro* and *in vivo* make them attractive vehicles for molecular therapy. Recently, a recombinant wild-type p53 adenoviral vector (Ad5CMV-p53) was generated. This provided us with an excellent candidate for investigation of the biological effects of the wild-type *p53* gene product on SCCHN cells bearing the mutated *p53* gene. Using a β-gal recombinant adenovirus, the gene transfer efficiency of SCCHN cells was established. Approximately 60% of SCCHN cells were positive after X-gal staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. This result coincided with the efficiency obtained in cells infected with Ad5CMV-p53 after immunostaining by using a monoclonal anti-p53 antibody. Our observed transduction efficiency was lower than that achieved in other cell lines tested, including HeLa, HepG2, LM2, and the human non-small cell lung cancer cell lines. This discrepancy could be due to a host of factors, including receptor variations and differences in membrane characteristics among the cell lines. Additionally, the transduction efficiency of SCCHN cells may have been underestimated by limitations of light microscopic analyses.

Ad5CMV-p53 mediated a high level of expression of the *p53* gene in SCCHN cells. Two *p53* mRNA species were detected in the Ad5CMV-p53-infected cells. The high level of 1.9-kilobase mRNA was derived from the transduced *p53* cDNA following infection with Ad5CMV-p53, indicating that the adenoviral vector is an efficient vehicle for gene delivery into human SCCHN cells. Moreover, the levels of endogenous 2.8-kilobase mRNA were higher in the transduced cells than in the controls, presumably due to the effect of wild-type *p53* gene product. This phenomenon of transcriptional

[4] Unpublished data.

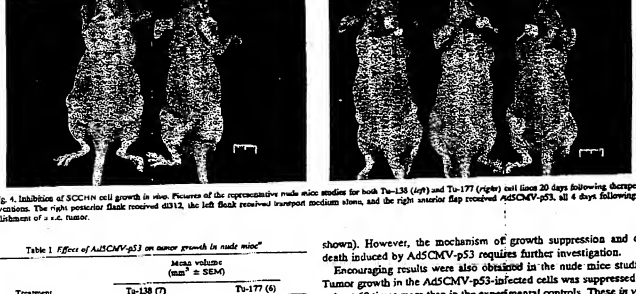


Fig. 4. Inhibition of SCCHN cell growth *in vivo*. Pictures of the representative nude mice studies for both Tu-138 (*left*) and Tu-177 (*right*) cell lines 20 days following therapeutic interventions. The right posterior flank received dl312, the left flank received transport medium alone, and the right anterior flap received Ad5CMV-p53, all 4 days following the establishment of s.c. tumor.

Table 1 *Effects of Ad5CMV-p53 on tumor growth in nude mice*[a]

Treatment	Mean volume (mm^3 ± SEM) Tu-138 (7)	Tu-177 (6)
Ad5CMV-p53	22.3 ± 14	13 ± 18
Ad5(dl312)	803 ± 300	533 ± 148
Medium	1207 ± 511	421 ± 143
Significance	*P*	*P*
p53[b]:dl312	0.03	0.02
p53:medium	0.04	0.03

[a] The cells were injected s.c. at 5×10^6 cells/flap. Tumor sizes were determined at day 20 after treatment. Numbers in parentheses, the number of animals evaluated.
[b] Ad5CMV-p53 is abbreviated as p53; dl312 is an abbreviation for Ad5(dl312).

autoregulation of the *p53* gene has been well documented in murine cell lines in which the wild-type *p53* can transactivate its own promoter and the mutant *p53* fails to regulate the *p53* promoter (24).

Due to the episomal property of adenoviral vectors, all the input DNA following infection with Ad5CMV-p53 is presumably degraded slowly throughout incubation. By using polymerase chain reaction-based detection techniques, DNA can be detected as late as 14 days postinfection (data not shown).

Western blot analysis demonstrated that there were few or no changes of p53 protein levels between mock- and replication-defective adenovirus-infected cells, whereas production of p53 protein was significant in Ad5CMV-p53-infected cells, suggesting that the exogenous *p53* mRNA was efficiently translated. Time course protein expression studies have shown protein expression to peak 3 days postinfection and progressively decline to still detectable Western blotting levels on day 15 (22). Functionally, these SCCHN cells transduced with wild-type p53 gene exhibited significant inhibition of growth *in vitro* as compared to the mock-infected and replication-defective cells, thus clearly illustrating that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth *in vitro* may be related to arrest of the G_1 cell cycle (18), apoptosis (19, 20), or induction of another tumor suppressor gene such as *WAF1/CIP1* (25). The induction of apoptosis is one of the several documented functions of wild-type *p53*. When Tu-138 and Tu-177 cells were infected with Ad5CMV-p53 at 100 plaque-forming units/cell, the characteristic apoptotic histomorphology, such as rounded-up cells and the formation of blebs, was apparent as early as 4 h after infection and was followed rapidly by cell death (data not shown). However, the mechanism of growth suppression and cell death induced by Ad5CMV-p53 requires further investigation.

Encouraging results were also obtained in the nude mice studies. Tumor growth in the Ad5CMV-p53-infected cells was suppressed by at least 60 times more than in the experimental controls. These *in vivo* results confirmed the *in vitro* s...ces of Ad5CMV-p53 on human SCCHN cells, suggesting that the wild-type p53 protein mediates a potentially therapeutic effect. Although the *in vivo* studies are in their infancy, they nevertheless portend the development of a model for gene therapy in SCCHN that uses p53 adenovirus as a therapeutic intervention. Information derived from such studies could be expanded in the development of other novel molecular therapies that use adenoviral vectors, not only in SCCHN but in other human cancers. Several critical questions remain unanswered. How should the insult from antibodies that may arise in animals or patients following viral treatment be alleviated? How safe is this virus in humans? The results of the preliminary studies justify further investigation of *in vivo* animal models as well as mechanisms through which wild-type *p53* regulates these *in vitro* and *in vivo* effects.

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T. E. Johnson, in preparation).
23. In reviewing unpublished studies from my laboratory (T. B. Johnson, unpublished data) involving smaller populations (no larger than 50 worms) and only thrice-weekly survival assessments, 14 of 19 comparisons show lower initial mortality rates for age-1 whereas only 4 of 19 show similar changes for the exponential Gompertz component. It thus seems that larger populations and more frequent assays are required to detect the effects described in this report.
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Table 1. Colony formation after transfection with wild-type and mutant p53 expression vectors. For each experiment, one or two 75-cm² flasks were transfected (*13*), and the total colonies counted after 3 to 4 weeks of selection in geneticin (0.8 mg/ml). Exp., experiment.

Cell line	Exp.	No. of geneticin-resistant colonies formed	
		pC53-SCX3 (mutant)	pC53-SN3 (wild-type)
SW837	1	754	66
	2	817	62
SW480	1	449	79
	2	364	26
RKO	1	1858	190
	2	1825	166
VACO 235	1	18	16
	2	26	28

Suppression of Human Colorectal Carcinoma Cell Growth by Wild-Type p53

SUZANNE J. BAKER, SANFORD MARKOWITZ, ERIC R. FEARON, JAMES K. V. WILLSON, BERT VOGELSTEIN*

Mutations of the p53 gene occur commonly in colorectal carcinomas and the wild-type p53 allele is often concomitantly deleted. These findings suggest that the wild-type gene may act as a suppressor of colorectal carcinoma cell growth. To test this hypothesis, wild-type or mutant human p53 genes were transfected into human colorectal carcinoma cell lines. Cells transfected with the wild-type gene formed colonies five- to tenfold less efficiently than those transfected with a mutant p53 gene. In those colonies that did form after wild-type gene transfection, the p53 sequences were found to be deleted or rearranged, or both, and no exogenous p53 messenger RNA expression was observed. In contrast, transfection with the wild-type gene had no apparent effect on the growth of epithelial cells derived from a benign colorectal tumor that had only wild-type p53 alleles. Immunocytochemical techniques demonstrated that carcinoma cells expressing the wild-type gene did not progress through the cell cycle, as evidenced by their failure to incorporate thymidine into DNA. These studies show that the wild-type gene can specifically suppress the growth of human colorectal carcinoma cells in vitro and that an in vivo–derived mutation resulting in a single conservative amino acid substitution in the p53 gene product abrogates this suppressive ability.

ONE COPY OF THE SHORT ARM OF chromosome 17, which harbors the p53 gene, is lost in many human tumors, including those of the colon and rectum (*1–3*). In the majority of human colon carcinomas with allelic deletions of chromosome 17p, the remaining p53 allele contains a missense mutation (*3, 4*). In addition to colorectal carcinomas, p53 gene mutations have also been found in conjunction with chromosome 17p allelic deletions in tumors of the brain, breast, lung, and bone (*4–6*). These studies are consistent with the hypothesis that the normal (wild-type) p53 gene product may function as a suppressor of neoplastic growth, and that mutation or deletion, or both, of the wild-type gene inactivates this suppression. This hypothesis has been supported by studies in rodent cells. For example, p53 alleles are often rearranged or mutated as a result of viral integration events in Friend virus–induced mouse erythroleukemias (*7*). Additionally, in transfection studies, the wild-type murine p53 gene has been shown to inhibit the transforming ability of mutant p53 genes in rat embryo fibroblasts (*8*). Other studies, however, have suggested that expression of the wild-type p53 gene product is necessary (not inhibitory) for cell growth (*9, 10*). Thus, the effect of wild-type and mutant p53 genes on cell growth may depend on the cell type examined. We now show that expression of the wild-type p53 gene in human colorectal carcinoma cells dramatically inhibits their growth. Moreover, a p53 gene mutant cloned from a human colorectal carcinoma was biologically inactive in this respect, as it was incapable of inhibiting such growth.

The colorectal carcinoma lines SW480 and SW837, which are representative of 75% of colon carcinomas, have each lost one copy of chromosome 17p (including the p53 gene), and the remaining p53 allele is mutated (*3, 4*). The SW837 line contains an arginine to tryptophan mutation at codon 248 (*4*). The SW480 line contains two point mutations, arginine to histidine at codon 273 and proline to serine at codon 309 (*4*). The substitutions at codon 248 and 273 are typical of those observed in human tumors, occurring within two of the four mutation "hot spots" (*4*). For the transfection studies, we constructed a vector, pCMV-Neo-Bam, engineered to contain two independent transcription units (*11*). The first unit comprised a cytomegalovirus (CMV) promoter/enhancer upstream of a site for insertion of the cDNA sequences to be expressed, and splice and polyadenylation sites to ensure appropriate processing. The second transcription unit included a herpes simplex virus (HSV) thymidine kinase promoter/enhancer upstream of the neomycin resistance gene, allowing for selection of transfected cells in geneticin (*11*). A wild-type p53 cDNA was inserted into pCMV-Neo-Bam to produce pC53-SN3. Similarly, a vector, pC53-SCX3, expressing a mutant cDNA from human colorectal tumor CX3, was also constructed. The only difference between pC53-SN3 and pC53-SCX3 was a single nucleotide (C to T) resulting in a substitution of alanine for valine at p53 codon 143 in pC53-SCX3 (*12*).

The constructs were transfected into SW837 and SW480 cells (*13*), and geneticin-resistant colonies were counted 3 weeks later. Cells transfected with pC53-SN3 formed five- to tenfold fewer colonies than those transfected with pC53-SCX3 in both recipient cell types (Table 1). In both

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SW837 and SW480 cells, the number of colonies produced by the expression vector pCMV-Neo-Bam (without a p53 cDNA insert) was similar to that induced by the pC53-SCX3 construct.

These results suggested that the wild-type p53 gene inhibited the clonal growth of both the SW837 and SW480 cell lines; however, a significant number of colonies formed after transfection of the wild-type construct. If wild-type p53 expression were truly inhibitory to cell growth, one would expect that no colonies would form or that p53 expression in the colonies that did form would be reduced compared to that produced with the mutant p53 cDNA construct. To evaluate this issue, we expanded independent SW480 and SW837 colonies into lines, and ribonuclease (RNase) protection analysis was performed to determine the amount of p53 mRNA expressed from the exogenously introduced sequences. Twelve of 31 lines (38%) derived from transfection with the pC53-SCX3 construct were found to express the exogenous mutant p53 mRNA. This percentage was consistent with results expected in human cells transfected with a vector containing two independent transcription units (*14*). In contrast, no expression of exogenous p53 wild-type mRNA was seen in any of 21 clonal lines established from either SW480 or SW837 cells transfected with the pC53-SN3 vector (Fig. 1A). These RNase protection results were supported by analysis of the exogenous p53 DNA sequences within the clones. All of the p53-expressing clones derived from the pC53-SCX3 transfection contained an intact copy of the exogenous p53 gene (Fig. 1B). In contrast, in all the clones derived from the pC53-SN3 transfection, the exogenous p53 sequences were deleted or rearranged (Fig. 1B).

The results from individual clones were further supported by the analysis of pooled clones, in which numerous colonies could be simultaneously assessed. Forty or more clones from two to three separate transfection experiments were pooled and analyzed approximately 3 weeks after transfection. RNase protection studies showed substantial expression of exogenous mutant sequences in the pooled clones, whereas expression of wild-type sequenees was not detectable (Fig. 2A). Results from Southern (DNA) blotting were consistent with the RNase protection studies, in that pooled colonies from the wild-type transfectants had no detectable unrearranged exogenous p53 sequences, in contrast to the intact p53 sequences in colonies derived from the mutant p53 cDNA expression vector (Fig. 2B).

The conclusions made from the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (Figs. 1A and 2A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells (*15*). However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected human cells expressed p53 protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). Although RKO cells did not contain a mutation within the susceptible p53 coding sequences (*16*), they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein (*15*).

Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells (*17*) revealed that approximately equal numbers of cells expressed wild-type and mutant protein 6 hours after transfection. A twofold difference was found at 24 hours, and this

Table 2. Immunocytochemistry and [^{3}H]thymidine incorporation of transfected RKO cells. To determine p53 expression, we split RKO cells into eight flasks and individually transfected them with either pC53-SCX3 or pC53-SN3 (*13*). At the indicated times after transfection, cells from each flask were fixed and stained with a monoclonal antibody to p53 protein (*17*). At least 1500 cells were counted for each determination. To determine [^{3}H]thymidine incorporation, we split RKO cells into duplicate flasks and individually transfected them with either pC53-SCX3 or pC53-SN3 (*13*). Forty-six hours after transfection, the cells were incubated with [^{3}H]thymidine for 2 hours, then fixed and stained with a monoclonal antibody to p53 protein (*17*). Evaluation of thymidine incorporation in the transfected cells was performed as previously described (*19*). At least 50 p53-expressing cells and 400 p53-nonexpressing cells (determined immunocytochemically with antibody to p53) were assessed for each determination of [^{3}H]thymidine uptake.

Plasmid	Percent of cells expressing p53 protein at				Percent of cells incorporating [^{3}H]thymidine in	
	6 hours	24 hours	48 hours	96 hours	p53 expressors	p53 nonexpressors
pC53-SCX3	2.0	11	4.3	2.0	24	31
pC53-SN3	1.9	5.2	0.3	0.2	1.7	33

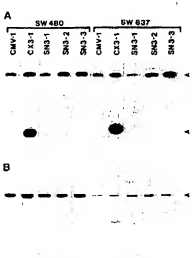

Fig. 1. (**A**) RNase protection analysis of transfected clonal lines. A labeled antisense p53 probe, which distinguishes between endogenous p53 mRNA and exogenous p53 mRNA, was hybridized with total cellular RNA from representative lines established from independent geneticin-resistant clones (*23*). After digestion with RNase A, the resulting hybridization products were separated by electrophoresis on denaturing polyacrylamide gels and autoradiographed (*24*). The labeled probe comprised nucleotides 1450 to 1788 relative to the p53 translation initiation site. Endogenous p53 mRNA included all of the p53 sequences represented in the labeled probe, so a 388-bp hybridization product (Endo.) was protected from RNase digestion. The exogenous p53 mRNA produced from the expression vector, however, only extended to nucleotide 1671; hybridization to exogenous p53 mRNA followed by RNase digestion therefore gave rise to a 221-bp fragment (Exo.). Clonal lines designated CMV, CX3, and SN3 were transfected with pCMV-Neo-Bam, pC53-SCX3, and pC53-SN3, respectively. (**B**) Southern blot analysis of transfected clonal lines. DNA from representative clonal lines (Fig. 1A) was digested with Bam HI, separated by electrophoresis on an agarose gel, transferred to nylon, and hybridized to a labeled p53 gene probe (*25*). The exogenous p53 gene from the expression vector was present in a 1.8-kb Bam HI fragment (Exo.) if it had not been rearranged in the cell. The endogenous p53 gene (Endo.) gave rise to a 7.8-kb Bam HI fragment.

difference increased with time (Table 2). These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (*18*). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours (*15*), supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

To obtain additional evidence that cells expressing p53 were inhibited in their growth potential, we examined the effect of p53 gene expression on DNA synthesis in transfected RKO cells. Forty-eight hours after transfection, RKO cells were labeled with [^{3}H]thymidine for 2 hours. The cells were subsequently fixed, immunocytochemically stained for the presence of p53 protein, and autoradiographed (*19*). The number of cells undergoing DNA replication was only slightly lower in cells producing exogenous mutant p53 protein than in cells that did not express any detectable p53 protein. Expression of the wild-type protein, however, dramatically inhibited the incorporation of thymidine (Table 2).

These results all suggested that wild-type p53 exerted an inhibitory effect on the growth of carcinoma cells in vitro. To evaluate whether this inhibitory effect was cell type–specific, we transfected colorectal epithelial cells derived from a benign tumor of the colon (the VACO 235 adenoma cell line). Previous studies have shown that most adenomas contain two copies of chromosome 17p and express wild-type p53 mRNA at concentrations similar to that of normal colonic mucosa (*1, 15*). Analogously, the p53 alleles of the VACO 235 cell line were sequenced and found to be wild type (*16*), and the expression of p53 mRNA was found to be similar to that of normal colorectal mucosa (*15*). In contrast to the results seen with SW480, SW837, and RKO cells, the pCS3-SN3 and pCS3-SCX3 constructs produced similar numbers of geneticin-resistant colonies after transfection of the VACO 235 line (Table 1). We considered, however, that the most definitive test for differential growth inhibition by wild-type versus mutant p53 genes involved analysis of exogenous p53 expression in pooled transfectants. Through such analysis, a large number of colonies could be examined simultaneously and the expression of exogenous mutant and wild-type p53 genes directly compared. Striking differences in the relative expression from the transfected genes were seen in all three carcinoma cell lines tested. VACO 235 transfectants, however, expressed similar amounts of exogenous p53 mRNA from either pCS3-SN3 (wild-type) or pCS3-SCX3 (mutant) p53 constructs (Fig. 2A).

In summary, our results suggest that expression of the wild-type p53 gene in colorectal carcinoma cell lines was incompatible with proliferation. The inhibitory effects of wild-type p53 were specific in two ways. First, a single point mutation in a p53 gene construct abrogated its suppressive properties as measured by three separate assays (colony formation, exogenous p53 expression in transfected clones, and thymidine incorporation). The CX3 mutant provided a control for gene specificity, as it contained only one conservative mutation, resulting in a substitution of one hydrophobic amino acid (alanine) for another (valine) at a single codon. Second, the growth-suppressive effect of the wild-type p53 construct was cell type–specific. Introduction of the wild-type vector into the VACO 235 adenoma cell line had no measurable inhibitory effect compared to the mutant p53 vector. There are several differences between the cell lines that could account for the differential effect of the introduced vectors. Regardless of the basis for the difference, the results with the VACO 235 cell line minimize the possibility that the wild-type p53 construct had some nonspecific, toxic effect on recipient cells; the effect was cell type–dependent.

The transfection and expression results of Table 1 and Fig. 2A suggest that cells at the premalignant stages of tumor progression (VACO 235) may be less sensitive to the inhibitory effects of wild-type p53 than malignant cells (SW480, SW837, and RKO). This hypothesis is consistent with previous results that suggest the wild-type p53 is less inhibitory to the growth of normal rat embryo fibroblasts than to their oncogene-transfected derivatives (*8*). This sensitivity may only be relative: expression of the wild-type gene at high concentrations might inhibit the growth of any cell type, including non-neoplastic cells, by overwhelming normal regulatory processes such as phosphorylation (*20, 21*). Genetic alterations that occur during the progression of colorectal tumors (*22*) may increase the sensitivity of cells to p53 inhibition, making wild-type p53 expression a key, rate-limiting factor for further tumor growth and expansion. At this point, and not before, mutations in the p53 gene would confer a selective growth advantage to cells in vivo, which would explain the frequent occurrence of p53 gene mutations and allelic loss only in the more advanced stages of colorectal tumorigenesis (*1, 22*).

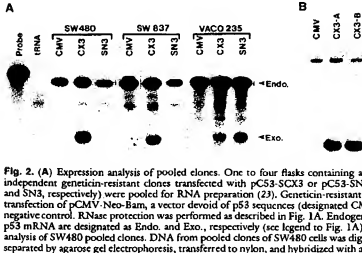

Fig. 2. (A) Expression analysis of pooled clones. One to four flasks containing a total of at least 40 independent geneticin-resistant clones transfected with pCS3-SCX3 or pCS3-SN3 (designated CX3 and SN3, respectively) were pooled for RNA preparation (*23*). Geneticin-resistant clones formed after transfection of pCMV-Neo-Bam, a vector devoid of p53 sequences (designated CMV), were used as a negative control. RNase protection was performed as described in Fig. 1A. Endogenous and exogenous p53 mRNA are designated as Endo. and Exo., respectively (see legend to Fig. 1A). **(B)** Southern blot analysis of SW480 pooled clones. DNA from pooled clones of SW480 cells was digested with Bam HI, separated by agarose gel electrophoresis, transferred to nylon, and hybridized with a labeled probe from the p53 gene as described (*23*). The lanes represent pooled clones from SW480 cells transfected with the following: lane CMV, pCMV-Neo-Bam; lanes CX3-A and CX3-B, pCS3-SCX3 (two independent pools); and lanes SN3-A and SN3-B, pCS3-SN3 (two independent pools). The 7.8-kb fragment from the endogenous p53 gene is indicated as Endo., and the 1.8-kb fragment from the exogenously introduced DNA is indicated as Exo.

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11. The expression vector pCMV-Neo-Bam was derived from plasmid BCMGNeo-nIL2 [H. Karasuyama, N. Yokoyama, T. Tada, *J. Exp. Med.* **169**, 13 (1989)] by excision of the human beta globin sequences and bovine papilloma virus sequences with Bam HI and Not I. Next, the interleukin 2 (IL-2) sequences present at the unique Xho I site were removed, and the Xho I site was changed to a Bam HI site by linker addition. The vector included CMV promoter/enhancer sequences, which could drive expression of the insert at the Bam HI site, and splicing and polyadenylation sites derived from the rabbit beta globin gene, which ensured proper processing of the transcribed insert in the cell. A pBR322 origin of replication and beta-lactamase gene facilitated growth of the plasmid in *Escherichia coli*. The plasmid conferred geneticin resistance through expression of the neomycin resistance gene under separate control of an HSV thymidine kinase promoter.
12. A 1.8-kb Xba I fragment, extending from nucleotide −130 to 1671 relative to the translation initiation site, was isolated from wild-type or CX3 cDNA clones (*3*). The fragment was blunt-ended with the Klenow fragment of DNA polymerase, ligated to Bam HI linkers, and cloned into the unique Bam HI site in the expression vector pCMV-Neo-Bam.
13. SW480 and SW837 were obtained from American Type Culture Collection (ATCC). RKO cells were obtained through the generosity of M. J. Bruttain. VACO 235 cells are described by J. K. V. Willson *et al.* [*Cancer Res.* **47**, 2704 (1987)]. For transfection, carcinoma cells at 30 to 60% confluence were incubated in a 75-cm² flask in 5 ml of Optimem (Gibco) with 5 μg of plasmid DNA and 30 μg of lipofectin [P. L. Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413 (1987)]. After 5 to 16 hours, the Optimem was replaced with Dulbecco's or McCoy's 5A medium containing 10% fetal calf serum. Selection in geneticin (0.8 mg/ml) began 36 to 48 hours after transfection for colony formation assays. Electroporation was used to transfect VACO 235 cells essentially as described by H. Potter, L. Weir, and P. Leder [*Proc. Natl. Acad. Sci. U.S.A.* **81**, 7161 (1984)].
14. Previous studies have shown that, in contrast to rodent cells, primate cells are able to integrate only a small amount of foreign DNA (approximately 6 kb), so that only 10 to 20% of clones selected for the expression of one transcription unit also contain the second unit in an intact form [F. Collabere-Garapin, M. Rhyiner, I. Stephany, P. Kourilsky, A. Garapin, *Gene* **60**, 279 (1986); J. H. J. Hoeijmakers, H. Odijk, A. Westerveld, *Exp. Cell Res.* **169**, 111 (1987); L. Mayne *et al.*, *Gene* **66**, 65 (1988); S. W. Dean, L. Kinch, H. R. Sykes, A. B. Lehmann, I. A. Wise, *Exp. Cell Res.* **183**, 473 (1989)].
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16. The p53 gene sequences in exons 5, 6, 7, 8, and 9 were examined essentially as described in (*4*). All previously noted point mutations in p53 genes have involved one of these exons (*see 3–5*).
17. Approximately 5×10^6 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against human p53 protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein [L. Banks, G. Matlashewski, L. Crawford, *Eur. J. Biochem.* **159**, 529 (1986)]. Ten to 20 randomly selected microscopic fields were analyzed per slide.
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19. Cells were grown for 2 hours in McCoy's 5A medium with 10% fetal calf serum and [^{3}H]thymidine (10 μCi/ml of 50 Ci/mmol, New England Nuclear). After immunocytochemical staining (*17*), slides were dehydrated in ethanol, dipped in NTB-2 emulsion (Kodak), and exposed for 2 weeks at 4°C. Autoradiographs were developed for 2 min in D-19 and stabilized in Rapid Fix (Kodak).
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25. DNA purification, restriction endonuclease digestion, electrophoresis, transfer, and hybridization were performed as described (*1, 3*). The hybridization probe was a 1.8-kb Xba I fragment of p53 cDNA (*12*).
26. We thank A. Preisinger, K. Molkentin, and J. Jackson for technical assistance. This work was supported by grants GM 07184, GM 07309, CA 43703, CA 45967, CA 43460, CA 51504, and CA 35494 from the NIH.

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A β_3 Integrin Mutation Abolishes Ligand Binding and Alters Divalent Cation–Dependent Conformation

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The ligand-binding function of integrin adhesion receptors depends on divalent cations. A mutant $\alpha_{IIb}\beta_3$ integrin (platelet gpIIb/IIIa) that lacks ligand recognition shows immunologic evidence of a perturbed interaction with divalent cations. This was found to be caused by a G→T mutation that resulted in an $Asp^{119} \rightarrow Tyr^{119}$ substitution in the β_3 subunit. This residue is proximal to bound ligand and is in a conserved region among integrins that are enriched in oxygenated residues. The spacing of these residues aligns with the calcium-binding residues in EF hand proteins, suggesting interaction with receptor-bound divalent cation as a mechanism of ligand binding common to all integrins.

CELL-CELL AND CELL-MATRIX ADHESIVE INTERACTIONS ARE ESSENTIAL TO DEVELOPMENT, INFLAMMATION, HEMOSTASIS, AND IMMUNE RECOGNITION. The integrins are a broadly distributed family of structurally related receptors that contribute to these adhesive reactions by recognition of a multiplicity of extracellular matrix protein ligands including laminin, collagens, fibrinogen, and bone sialoprotein (*1*). In addition, integrins participate in cell-cell interactions by recognition of integral membrane protein ligands including the intercellular adhesion molecules ICAM-1 and ICAM-2 and vascular cell adhesion molecule–1 (VCAM-1) (*2*). Although the integrins differ in ligand recognition specificity, a requirement for millimolar concentrations of physiologic divalent cations is common to the primary recognition function of all integrins (*3*). This dependence of function on divalent cations can be attributed to a low-affinity divalent cation–binding site within the integrins, because millimolar Ca^{2+} or Mg^{2+} can modulate the conformation of a prototype integrin, platelet membrane glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$ also known as gpIIb/IIIa), which is detectable by a monoclonal antibody (MAb) PMI-1 (*4*). Loss of the epitope recognized by this MAb directly correlates with the capacity of $\alpha_{IIb}\beta_3$ to bind fibrinogen. The Cam variant of Glanzmann's thrombasthenia (*4*) is an autosomal recessive hereditary disorder of $\alpha_{IIb}\beta_3$ that is associated with the inability of this integrin to recognize macromolecular (*4*) or synthetic peptide (*5*) ligands. In addition, divalent cations do not regulate the expression of the PMI-1 epitope in Cam platelets (*4*). These characteristics indicate that the presumptive mutation in the Cam receptor leads to defects in binding of both divalent cations and primary ligands. To elucidate the structural basis of integrin function, we identified the point mutation in $\alpha_{IIb}\beta_3$ that causes the Cam variant of Glanzmann's thrombasthenia.

Total RNA was isolated from platelets of normal donors and two affected siblings with Cam variant. For initial sequencing, we

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Biochemistry

Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line

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ABSTRACT **A wild-type p53 gene under control of the metallothionein MT-I promoter was stably transfected into human colon tumor-derived cell line EB. Repeated inductions of the metallothionein wild-type p53 gene with zinc chloride results in progressive detachment of wild-type p53 cells grown on culture dishes. Examination at both the light and electron microscopic level revealed that cells expressing wild-type p53 developed morphological features of apoptosis. DNA from both attached and detached cells was degraded into a ladder of nucleosomal-sized fragments. Expression of wild-type p53 inhibited colony formation in soft agar and tumor formation in nude mice. Furthermore, established tumors in nude mice underwent regression if wild-type p53 expression was subsequently induced. Regressing tumors showed histological features of apoptosis. Thus, regression of these tumors was the result of apoptosis occurring *in vivo*. Apoptosis may be a normal part of the terminal differentiation program of colonic epithelial cells. Our results suggest that wild-type p53 could play a critical role in this process.**

p53 was originally discovered as a consequence of its association with simian virus 40 (SV40) large tumor antigen (1, 2). Subsequently, it was shown that mutant p53 could immortalize primary fibroblasts (3, 4) and, in collaboration with mutant ras, could transform them (5, 6). p53 mutations have been found in virtually all types of human tumors examined (7, 8) including tumors of the lung (9, 10), breast (11), liver (12, 13), and colon (14, 15). Homozygosity is observed in most cases of p53 gene mutation, indicating that the wild-type (wt) allele is lost through deletion (16). The conclusion that wt p53 behaves as a recessive oncogene in tumors is supported by cotransfection experiments in which wt p53 suppresses transformation by mutant p53 with a mutant ras (17, 18).

wt p53 stops growth in most transformed cells into which it has been introduced (19, 20). Furthermore, overexpression of wt p53 causes cell cycle arrest near the G_1–S boundary (21–24). *In vitro* replication of SV40 DNA is inhibited by wt p53 (25, 26). Recently, Bargonetti *et al.* (27) have demonstrated that wt p53, but not mutant p53, binds to a region of SV40 DNA adjacent to the origin of replication (27). In addition, it has been reported that a wt p53–gal4 DNA binding domain fusion protein can promote transcription, suggesting that p53 can act as a positive transcription factor (28, 29). Thus, it is possible that p53 regulates a set of genes playing a role in the passage from G_1 to S (16).

We wished to investigate further the function of wt p53 by studying the effects of wt p53 reexpression in human colon tumor cell line EB (30). Induced expression of the transfected wt p53 gene resulted in apoptosis *in vitro*, prevented colony formation in soft agar, and inhibited tumor growth in nude mice. Established tumors in nude mice underwent regression upon induction of the wt p53 gene.

MATERIALS AND METHODS

Cell Culture. EB cells (30) and the stably transfected clones derived from them were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (GIBCO).

Metallothionein (MT) p53 Construction, Transfection, and Selection of Clones. MT p53 was constructed by blunt-end ligating a 212-nucleotide *Sac I/Bgl* II fragment of the rat MT-1 promoter (Δ156) (31) to the filled-in *EcoRI* site at the 5′ end of a human wt p53 cDNA clone (32). EB cells were cotransfected with MT p53 and SV-Neo (33) plasmid DNAs by the calcium phosphate procedure. Stable clones were selected for their resistance to G418 (800 μg/ml) and clones were isolated with cloning cylinders. They were then screened for the presence of the wt p53 gene at second passage by PCR (34) using the oligonucleotides IN4AL (5′-AGA GGA ATT CTT CCT CTT CCT GCA GTA C-3′) and IN7AL (5′-TTG AGG ATC CCA AGT GGC TCC TGA CCT G-3′). The amplified DNA fragments are 1100 base pairs (bp) for the endogenous p53 gene and 385 bp for the transfected cDNA construct. Conditions of the 30-cycle PCR were 94°C for 1 min, 63°C for 1.5 min, and 72°C for 2 min in standard PCR buffer conditions (34).

DNA, RNA, and Protein Extraction and Analysis. RNA was extracted by the guanidine isothiocyanate/acid phenol method (35). RNA (10 μg) was treated with glyoxal and separated on a 0.8% agarose gel (36). RNA in the gel was transferred to a GeneScreen membrane (NEN) and then hybridized to a p53 cDNA probe (32) labeled by the random primer method (37) in 50% formamide/5× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate)/50 mM sodium phosphate, pH 6.5/0.5% SDS/denatured salmon sperm DNA (100 μg/ml). DNA and protein were prepared by standard methods (38). DNA was separated on a Tris acetate-buffered gel containing 1.5% agarose. The DNA was then transferred to a GeneScreen membrane (NEN) and hybridized with total human DNA labeled by the random primer method as described above for Northern blot analysis. Western blot analysis was by standard procedures with the pAB240 antibody (39).

MT Promoter Induction. Induction of the wt p53 gene was achieved with either 6 μM cadmium chloride (for screening of stable clones; see Fig. 1*e*) or 100 μM zinc chloride (for all other experiments). Tissue culture medium was not changed during multiple inductions with zinc chloride. When cells were injected into nude mice, the area of injection was marked. For induction of wt p53 in nude mice tumors, zinc chloride [100 μM in phosphate-buffered saline (PBS); 0.4 ml] was injected subcutaneously in the region outside of the marked area, thus avoiding the tumor.

RESULTS

Characterization of MT wt p53 Clones. A wt p53 gene under the control of an inducible promoter was used to avoid

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Abbreviations: wt, wild type; MT, metallothionein; SV40, simian virus 40.

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growth inhibition during establishment of stably transfected cell lines (19, 21–24). A wt p53 cDNA (32) was ligated to a truncated rat MT promoter MT-1 (Δ156), which has been shown to have very low basal activity and yet be strongly inducible with metal ions (31). This construct was cotransfected with SV-Neo (33), as a selectable marker into the EB cell line (30). G418-resistant clones were isolated and tested at second passage for the presence of the wt p53 DNA by PCR. Expression levels of the positive clones were analyzed by Northern blotting. Shown in Fig. 1*a* are the results obtained with clones EB-1, -2, and -5, both uninduced and induced with 6 μM cadmium chloride. All three clones have high levels of a 2-kilobase (kb) p53 mRNA, which is derived from the transfected p53 gene (Fig. 1*a*). This mRNA was undetectable in the uninduced cells, even on long exposures (data not shown). Note also the absence of the 2.4-kb p53 mRNA expected from the endogenous p53 gene (Fig. 1*a*). Southern blot analysis of the EB p53 gene did not reveal any gross alterations in the structure of the gene (data not shown).

Since the MT promoter has been characterized primarily in single induction experiments, we wished to determine whether sustained high levels of wt p53 mRNA could be obtained by daily stimulation of the MT-1 promoter with zinc chloride. This is of obvious importance if a minimal amount of the p53 protein must be maintained throughout the cell cycle to achieve cell cycle arrest (21–24). We examined the amount of p53 mRNA present 4, 8, 24, and 48 h after a single induction with zinc chloride (Fig. 1*b*). Maximal expression was elicited 4 h after induction (Fig. 1*b*) (40). p53 mRNA was still detectable 24 h after a single induction. Subsequent inductions of the introduced wt p53 gene yielded higher levels of wt p53 mRNA than the initial induction (Fig. 1*b*, compare lanes 1× +4h with 2× +4h). The significant increase in mRNA levels upon repeated induction may be the consequence of first induction recruitment of transcription factors that remain in place, resulting in the increased efficiency of subsequent inductions. These results demonstrate that sustained high levels of p53 mRNA could be achieved in our transfected clones by daily inductions with 100 μM zinc chloride.

Western blot analysis of uninduced and induced EB and EB-1 cells is shown in Fig. 2. p53 protein is readily detectable 4 h after a single induction with zinc chloride. Also of interest is that 24 h after a second induction with zinc chloride there is still a significant level of p53 protein. Thus, the mRNA detected by the Northern blot experiments described above is functional. Note the absence of p53 protein in uninduced

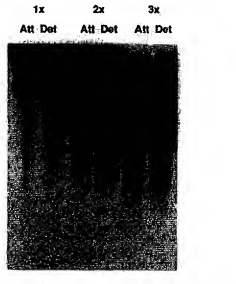

FIG. 2. Time course of DNA degradation in EB-1 after induction of wt p53 with zinc chloride. Attached (lanes Att) and detached (lanes Det) refer to cells that did or did not adhere to the culture dish at the time of cell harvest. 1×, 2×, and 3× refer to the number of inductions by zinc chloride. Cells were harvested 4 h after each induction with zinc chloride. Approximately equivalent amounts of DNA were loaded in each lane. Arrows indicate positions of nucleosomal multimers.

EB-1 cells and in EB cells with and without zinc chloride induction.

Induction of wt p53 Expression Is Accompanied by Apoptosis. While preparing RNA for the experiments described above, we noticed that the cells of the three wt p53 EB clones detached progressively from the plates over a 4-day period and eventually died. Approximately 10% of the cells detached on day 1, 30% detached on day 2, 50% detached on day 3, and 90% detached on day 4. The parental cell line continued to grow normally in the presence of zinc chloride with a doubling time of ≈45 h (P.S. and R.B., unpublished results). No gross differences in the percentage of cells detaching from the plates were observed for the three wt p53 clones. Cell death can occur by necrosis or apoptosis (41). There are several accepted criteria that can be used to distinguish between these two possibilities—namely, histo-

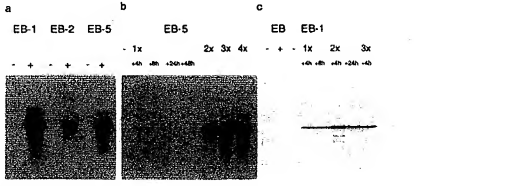

FIG. 1. Analysis of p53 mRNA and protein expression of MT wt p53 clones. (*a*) Ten micrograms of total RNA was analyzed for each clone by Northern blotting. RNA was prepared from cells either uninduced (lanes −) or induced (lanes +) with 6 μM $CdCl_2$ for wt p53 expression. (*b*) EB-5 cells were induced with 100 μM zinc chloride from one to four times. RNA was prepared at the time indicated after induction. 1× +4h indicates that the cells were induced once and that the RNA was prepared 4 h after induction. Subsequent inductions (2×, 3×, and 4×) were at 24-h intervals. (*c*) Western blot analysis of p53 in EB and EB-1. Protein extracts were prepared at the times indicated on the figure and were analyzed on 12% polyacrylamide gels. paB240 was used as the first antibody. Detection was with an alkaline phosphatase-conjugated second antibody.

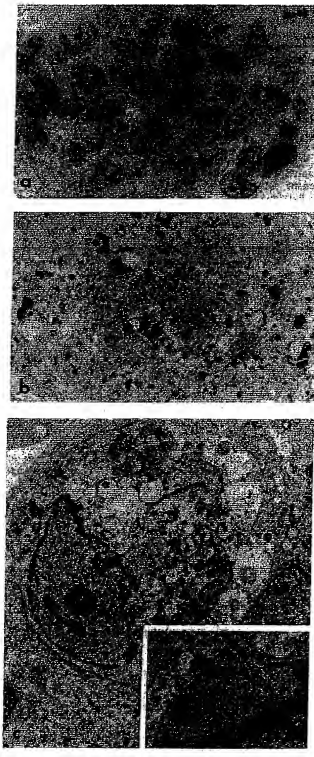

FIG. 3. Histology of induced and uninduced EB cell clones. (*a*) Histology of uninduced EB-1 cells grown *in vitro*. (*b* and *c*) Histology and electron micrographs of EB-1 cells after two inductions with 100 μM zinc chloride. (*Inset*) Leakage of chromatin from the nucleus is shown. (*a*, ×2400; *b*, ×2850; *c*, ×5900; *Inset*, ×8100.)

logical morphology and pattern of DNA degradation. We thus analyzed the state of the DNA in the parental cell line EB and in the transfected cell line EB-1 growing in the presence of zinc chloride. Parental EB cells contained only high molecular weight DNA (data not shown). On the other hand, DNA from cells expressing wt p53 underwent progressive degradation yielding a ladder of DNA fragments corresponding to multimers of nucleosomal-sized DNA (Fig. 2). This pattern of degradation is typical of apoptosis (41). It was striking that after only 4 h of metal ion stimulation, DNA degradation was already evident in the detached cell fraction. Thus, a small proportion of the cells underwent apoptosis very rapidly. After three consecutive inductions, the DNA was heavily degraded in both the attached and detached fractions. Morphological examination of EB-1 cells expressing wt p53 at the light and electron microscopic levels demonstrated typical features of cells undergoing apoptosis: condensation of chromatin in crescentic caps adjacent to the nuclear membrane, incomplete nuclear membranes, and translucent cytoplasmic vacuoles (Fig. 3 *a–c*).

Table 1. Suppression of tumor formation in nude mice

Cell line	Uninduced	Induced*
EB†	4/4	2/2
EB-1	4/4	0/2
EB-2	4/4	0/2
EB-5	4/4	0/2

Each nude mouse was injected with 5×10^6 cells.
*Zinc chloride (100 μM) was administered daily for ≈3 weeks starting 3 days after subcutaneous injection of cells into mice.
†Parental cell line.

Inhibition of Tumor Formation in Nude Mice by wt p53. Colony formation in soft agar and tumor formation in nude mice are criteria for tumorigenicity. All wt p53 clones, as well as the parental cell line, yielded colonies in soft agar at frequencies of 1–1.5%. Induction of the wt p53 gene completely abolished colony formation in all three cell lines—EB-1, -2, and -5 (data not shown). Colony formation in the parental cell line EB was unaffected by the presence of zinc chloride in soft agar.

Tumor growth in mice injected with the parental cell line proceeded normally, even in mice injected daily with zinc chloride. Tumors were obtained in nude mice injected with the three wt p53 clones only in the absence of zinc induction (4/4 in all cases; Table 1). When zinc chloride was administered daily, starting 3 days after injection of the cells, no tumors developed (0/2 mice for each clone). Thus, wt p53 expression prevented tumor formation in nude mice.

Regression of Established Tumors with wt p53 Expression. We further analyzed the effect of wt p53 induction on several established EB-1 tumors. Six weeks after injecting 5×10^6 cells, palpable tumors were present. We then induced wt p53 expression for a period of 3 weeks. This resulted in tumor regression, which was accompanied by morphological evidence of apoptosis (compare Fig. 4*a* and Fig. 4 *b* and *c*). The presence of scattered single necrotic cells is characteristic of apoptosis (41). The residual tumor mass comprised mainly neutrophils and scavenging macrophages (41). Induction of wt p53 for 2 months in established tumors resulted in total elimination of histologically detectable tumor cells at the site of cell injection (data not shown). Daily zinc chloride injections had no effect on established EB tumors (data not shown). Thus, expression of wt p53 in EB cells results in apoptosis both *in vitro* and *in vivo*.

DISCUSSION

The present experiments show that expression of wt p53 in the colon tumor-derived cell line EB induces apoptosis in cells grown *in vitro*, prevents colony formation in soft agar, prevents tumor growth in nude mice, and induces regression

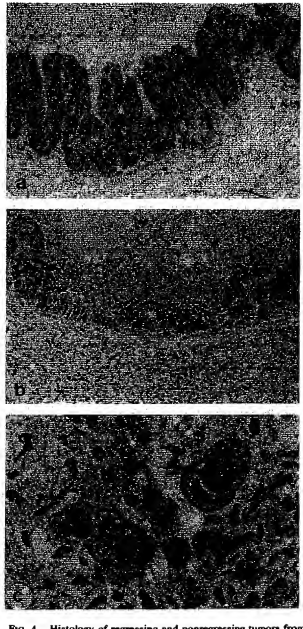

FIG. 4. Histology of regressing and nonregressing tumors from nude mice injected with EB-1 cells. (*a*) Control uninduced EB-1 tumors. (*b* and *c*) EB-1 tumors in which wt p53 was induced with zinc chloride. Tumors were allowed to form for ≈6 weeks. Induction of wt p53 by subcutaneous injections of 100 μM zinc chloride was initiated and continued daily for 3 weeks. Animals were sacrificed and dissected, and tumors were prepared for histological examination. Note the presence in c of several apoptotic cells. (*a* and *b*, ×750; *c*, ×2850.)

of established nude mice tumors *in vivo* by induction of apoptosis.

The generally accepted criteria for apoptosis—namely, nucleosomal DNA ladders and morphological features—are both observed when wt p53 expression is induced in EB cells. Zinc chloride, the metal inducer used in the present experiments, does not induce apoptosis in the parental cell line. In fact, the endonuclease responsible for the nucleosomal ladder observed in apoptosis is known to be inhibited by zinc chloride, although at considerably higher concentrations (42). Apoptosis of cells in culture was quite rapid, being essentially complete after 4 days of wt p53 induction. We further demonstrate that established tumors in nude mice regress upon induction of wt p53 expression. Apoptosis is observed in this situation as well. There is a large difference in the rate at which apoptosis proceeds in the tumor relative to cells in culture. Established tumors take ≈2 months to complete apoptosis. This may be due to a difference in growth rate of the cells in the tumor relative to cells in culture or to accessibility to the inducer zinc chloride. We feel that the first is more likely, since we have observed that cells in culture undergo apoptosis more rapidly when they are grown at low density (R.B. and P.S., unpublished observations).

Recently, Oren and co-workers (43) have reported that expression of a murine p53 mutant that is temperature sensitive for transformation induces apoptosis in the myeloid leukemic cell line M1 at temperatures at which p53 is predominantly in the wt conformation. Both EB and M1 cells are deficient in endogenous p53 expression. Whether this is of significance to induction of apoptosis by wt p53 is not known. wt p53 has been expressed in a variety of cell types, including colon tumor cell lines (19); yet only this report and that of Oren and co-workers describe induction of apoptosis. The rareness of the observation suggests that the cell type and state of differentiation before transformation may be important factors determining whether or not wt p53 expression results in apoptosis.

EB cells give rise to well-differentiated tumors (Fig. 4*a*) (44). They form pseudo-crypts and tight cell junctions, structures reminiscent of the normal colon. Cells of the colonic mucosa derive from stem cells located at the base of crypts. After division of the stem cells, daughter cells stop dividing and enter a phase of terminal differentiation as they migrate to the surface of the mucosa, such that there is a gradient of differentiation from the base of the crypt to the mucosa. Several days after reaching the surface of the mucosa, the terminally differentiated cells are sloughed off (45). It is possible that this detachment occurs as a consequence of apoptosis, which has been demonstrated to occur in the small intestine in response to chemical agents (46). HL-60 cells, a promyelocytic leukemic cell line, undergo apoptosis when induced to differentiate (47).

Apoptosis has been studied in a large variety of biological systems. Information concerning the relationship between cell cycle stage and apoptosis is, however, quite meager. Chemotherapeutic drugs, such as cisplatin and etoposide, induce apoptosis after arrest in G_2 (48). Mouse embryo cells, upon epidermal growth factor deprivation (49), and immature T cells treated with anti-T-cell receptor antibody (50) undergo apoptosis. The latter cases are associated with G_1 arrest. Although the examples are limited, it is provocative to consider that wt p53-induced apoptosis in our colon cell lines also occurs after an arrest in G_1.

We would like to thank David Lane for generously supplying p53 antibodies and Lionel Crawford for the wt p53 cDNA plasmid. Alain Zweibaum is to be thanked for kindly providing the EB cell line. We are grateful to H. Diggelmann and K. Iggo for their careful reading of the manuscript. In addition, we thank R. Iggo for performing the Western blot. We wish also to thank Janine Bamat for both electron and light microscopy and for all of the micrographic photography. This work was supported by grants from the Swiss National Science Foundation (P.S. and B.S.). The oligonucleotides used were synthesized on a PCR mate (Applied Biosystems) provided by funds from Sandoz (P.S.).

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Differential effects of butyrate derivatives on human breast cancer cells grown as organotypic nodules in vitro and as xenografts in vivo.

Planchon P, Magnien V, Beaupain R, Mainguene C, Ronco G, Villa P, Brouty-Boye D

Institut d'Oncologie Cellulaire et Moleculaire Humaine, Hopital Avicenne, Bobigny, France.

The antiproliferative and cytodifferentiating effects of a new stable butyric derivative, monobut-3, were compared using human MDA-MB-231 breast cancer cells grown in three dimension as either in vitro tumor nodules or in vivo xenograft tumors. In in vitro tumor nodules, monobut-3 exhibited marked growth inhibitory effects consistent with the results obtained in monolayer cell cultures. Some functional cell differentiation was also detected in treated nodules. In in vivo xenografts, monobut-3 significantly decreased MDA-MB-231 tumor take but did not affect the rate of tumor growth. No difference was noted in the histological characteristics of the xenografts between untreated and treated mice. Moreover, once monobut-3 treatment was discontinued, tumor growth rapidly resumed in tumor-free animals. The decreased efficacy of monobut-3 in in vivo MDA-MB-231 xenografts as compared to in vitro tumor nodules indicates that factors related to host environment may still limit the clinical effectiveness of this compound.

PMID: 1296809, UI: 93208321

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Pharmacodynamics of cisplatin in human head and neck cancer: correlation between platinum content, DNA adduct levels and drug sensitivity in vitro and in vivo.

Welters MJ, Fichtinger-Schepman AM, Baan RA, Jacobs-Bergmans AJ, Kegel A, van der Vijgh WJ, Braakhuis BJ

Toxicology Division, TNO Nutrition and Food Research Institute, Zeist, The Netherlands.

Total platinum contents and cisplatin-DNA adduct levels were determined in vivo in xenografted tumour tissues in mice and in vitro in cultured tumour cells of head and neck squamous cell carcinoma (HNSCC), and correlated with sensitivity to cisplatin. In vivo, a panel of five HNSCC tumour lines growing as xenografts in nude mice was used. In vitro, the panel consisted of five HNSCC cell lines, of which four had an in vivo equivalent. Sensitivity to cisplatin varied three- to sevenfold among cell lines and tumours respectively. However, the ranking of the sensitivities of the tumour lines (in vivo), also after reinjection of the cultured tumour cells, did not coincide with that of the corresponding cell lines, which showed that cell culture systems are not representative for the in vivo situation. Both in vitro and in vivo, however, significant correlations were found between total platinum levels, measured by atomic absorption spectrophotometry (AAS), and tumour response to cisplatin therapy at all time points tested. The levels of the two major cisplatin-DNA adduct types were determined by a recently developed and improved 32P post-labelling assay at various time points after cisplatin treatment. Evidence is presented that the platinum-AG adduct, in which platinum is bound to guanine and an adjacent adenine, may be the cytotoxic lesion because a significant correlation was found between the platinum-AG levels and the sensitivities in our panel of HNSCC, in vitro as well as in vivo. This correlation with the platinum-AG levels was established at 1 h (in vitro) and 3 h (in vivo) after the start of the cisplatin treatment, which emphasizes the importance of early sampling.

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Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma in vitro and in vivo.

Vingerhoeds MH, Steerenberg PA, Hendriks JJ, Dekker LC, Van Hoesel QG, Crommelin DJ, Storm G

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands.

This paper deals with the utility of immunoliposomes for the delivery of doxorubicin (DXR) to human ovarian carcinoma cells in vitro and in vivo. We aimed to investigate whether immunoliposome-mediated targeting of DXR to ovarian cancer cells translates in an enhanced anti-tumour effect compared with that of non-targeted DXR liposomes (lacking the specific antibody). Target cell binding and anti-tumour activity of DXR immunoliposomes were studied in vitro and in vivo (xenograft model of ovarian carcinoma). In vitro we observed that target cell binding and cell growth inhibition of DXR immunoliposomes is superior to that of non-targeted DXR-liposomes. However, in vivo, despite the efficient target cell binding and good anti-tumour response of DXR-immunoliposomes, no difference in anti-tumour effect, compared with non-targeted DXR-liposomes, could be determined. The results indicate that premature DXR leakage from immunoliposomes occurring before the actual target cell binding and subsequent DXR association with the tumour cells, explains why no significant differences in anti-tumour activity between DXR-immunoliposomes and non-targeted DXR-liposomes were observed in vivo.

PMID: 8855969, UI: 97008852

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The effect of high dose vitamin A on the morphology and proliferative activity of xenograft lung and head and neck cancer.

Mourad WA, Bruner JM, Vallieres E, McName C, Alabdulwahed S, Scott K, Oldring DJ

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In vitro studies have suggested that vitamin A lowers invasive potential of squamous cell carcinoma. Epidemiological data have also indicated that high dose vitamin A may improve survival in patients with previously resected lung carcinoma. To our knowledge, no studies have attempted to test the in vivo effect of vitamin A on the morphology and growth rate of lung and head and neck cancer. Freshly resected tumor cell suspensions were obtained by ex vivo fine needle aspiration and injected subcutaneously in duplicate in athymic male nude mice. Two to six weeks post-engraftment tests and controls were separated for each xenograft. Mice with test xenografts were given water soluble vitamin A (Aquasol ATM, Astra pharmaceutical, Westborough, MA, U.S.A) at a dose of 10,000 U/Kg/day intraperitoneally for 6 to 10 weeks (median 8 weeks). One to two hours prior to sacrifice bromodexouridine (BrdU) was injected intraperitoneally to assess the S-phase fraction in both test and control xenografts. Blood vitamin A levels in test and control animals were measured after sacrifice using high performance liquid chromatography (HPLC). Sections of test and control xenografts were routinely stained to assess morphologic differentiation and mitotic counts. Unstained sections of xenografts were immunostained by the antibody to BrdU to test for BrdU labeling index (BLI) reflecting S-phase fraction (SPF) and also by the MIB-1 antibody to assess proliferative activity. Eighteen tumors were studied. These included 9 squamous cell carcinomas of the lung, 5 squamous cell carcinomas of the head and neck, and 4 adenocarcinomas of the lung. Blood levels of vitamin A in test animals were 7 to 23 times those of the control animals (median 13 times). Neovascularization of the xenografts was seen in all cases. The morphology and mitotic activity of the test and control xenografts showed no significant difference. SPF and proliferative activity measured by BrdU and MIB-1 immunolabelling respectively showed no significant difference between test and control xenografts. Our study suggests that there is no significant in vivo effect of high dose vitamin A on the morphology and growth rate of xenografted non small cell carcinoma of the lung or squamous cell carcinoma of the head and neck.

1 : *Int J Oncol* 2000 Mar;16(3):599-610 Related Articles, Books

Transforming growth factor-beta and response to anticancer therapies in human liver and gastric tumors in vitro and in vivo.

Liu P, Menon K, Alvarez E, Lu K, Teicher BA

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Liver cancer and gastric cancer are the most common solid tumors worldwide. Transforming growth factor-beta (TGF-beta) production and lack of response to TGF-beta growth inhibitory effects have been associated with tumor progression and therapeutic resistance. HepG2, Hep3B, and SK-HEP-1 human liver cancer lines produce 3, 5.7, and 2.5 ng TGF-beta1; 1.4, 2, and 4 ng TGF-beta2 and 0.15, 0.2 and 0.22 ng TGF-beta3 per 107 cells (24 h). Expression of the TGF-beta type I receptor is 20x, 1x, and 0.6x the level in mink lung MvLu1 cells in the HepG2, Hep3B, and SK-HEP-1 cells, respectively. HepG2 and Hep3B cells do not express the TGF-beta type II receptor while SK-HEP-1 cells express 7x the level found in mink lung MvLu1 cells. Hs 746T, KATO III, RF-1, and RF-48 human gastric cancer cell lines produce 12. 5, 0.35, 0.4, and 0.4 ng TGF-beta1; 2.6, 0.95, 0.5, and 0.52 ng TGF-beta2 and 0.42, 0.17, 0.12, and 0.14 ng TGF-beta3 per 107 cells (24 h). Expression of TGF-beta type I receptor is 0.7x, 0.7x, 0.8x, 0.6x the level in mink lung MvLu1 cells in the Hs 746T, KATO III, RF-1 and RF-48 cells, respectively. KATO III cells are lacking in the TGF-beta type II receptor while Hs 746T, RF-1 and RF-48 cells express 10x, 0.8x, and 1x the levels in mink lung MvLu1 cells. The IC50 for TGF-beta1 is >>10 ng/ml in all of these lines except RF-48 where TGF-beta1 is mitogenic. The response of the cell lines to radiation, doxorubicin, mitomycin C, cisplatin, 5-fluorouracil, methotrexate, and gemcitabine showed that SK-HEP-1 was the most drug resistant liver cancer cell line and KATO III was the most drug resistant gastric cancer cell line. Overall, there was no correlation between TGF-beta secretion in cell culture and sensitivity of the cells to anticancer agents. Increased TGF-beta1 levels were detectable in the plasma of nude mice bearing Hep3B and Hs 746T xenografts. Those tumors which secreted greater amounts of TGF-beta were more therapeutically resistant in vivo.

PMID: 10675495, UI: 20141554

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Tumour-growth suppression in nude mice by a murine monoclonal antibody: factors hampering successful therapy.

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The murine MAb C215 has been shown to mediate ADMMC in vitro and to have a tumour-growth-suppressive effect on xenografted COLO 205 human colocarcinoma cells in nude mice. To overcome the limitations of MAb therapy, it is necessary to understand the underlying mechanisms of tumour-growth suppression. In the present work, we have used C215 to define the importance of different parameters involved in tumour therapy with murine IgG2a antibodies. The results show that there exists a period of roughly 2 days after inoculation into animals during which the tumour cells are sensitive to an inhibitory antibody-mediated effect. After this initial period, the in-vivo sensitivity of tumour cells to antibody-mediated inhibition is much reduced. Tumour cells can remain "dormant" and, despite ongoing antibody treatment, develop into tumours with a reduced growth rate, which is not caused by outgrowth of antigen-deficient tumour cells. Finally, a pronounced dependence of antibody-mediated tumour suppression on antibody dose was observed.

PMID: 1708368, UI: 91210009

Transfer of Genes to Humans: Early Lessons and Obstacles to Success

Ronald G. Crystal

Enough information has been gained from clinical trials to allow the conclusion that human gene transfer is feasible, can evoke biologic responses that are relevant to human disease, and can provide important insights into human biology. Adverse events have been uncommon and have been related to the gene delivery strategies, not to the genetic material being transferred. Human gene transfer still faces significant hurdles before it becomes an established therapeutic strategy. However, its accomplishments to date are impressive, and the logic of the potential usefulness of this clinical paradigm continues to be compelling.

Human gene transfer is a clinical strategy in which the genetic repertoire of somatic cells is modified for therapeutic purposes or to help gain understanding of human biology (*1*, *2*). Essentially, gene transfer involves the delivery, to target cells, of an expression cassette made up of one or more genes and the sequences controlling their expression. This can be carried out ex vivo in a procedure in which the cassette is transferred to cells in the laboratory and the modified cells are then administered to the recipient. Alternatively, human gene transfer can be done in vivo, in a procedure in which the expression cassette is transferred directly to cells within an individual. In both strategies, the transfer process is usually aided by a vector that helps deliver the cassette to the intracellular site where it can function appropriately (*1*, *2*).

Once considered a fantasy that would not become reality for generations, human gene transfer moved from feasibility and safety studies in animals to clinical applications more rapidly than expected by even its most ardent supporters (*1*–*3*). It is not the purpose of this review to detail all human protocols that have been proposed, but to use examples from the available information regarding ongoing human trials (*3*) to define the current status of the field.

How Is Human Gene Transfer Carried Out?

The choice of an ex vivo or in vivo strategy and of the vector used to carry the expression cassette is dictated by the clinical target. The vector systems for which data are available from clinical trials (retroviruses, adenoviruses, and plasmid-liposome complexes) transfer expression cassettes through different mechanisms and thus have distinct advantages and disadvantages for different applications (*1*, *2*).

Vectors. Replication-deficient, recombinant retrovirus vectors can accommodate up to 9 kb of exogenous information (Fig. 1). Retroviruses transfer their genetic information into the genome of the target cell, and thus, theoretically, the target cell's genotype is permanently modified (*1*, *2*, *5*). This is an advantage when treating hereditary and chronic disorders, but it has risks, including the potential for toxicity associated with chronic overexpression or insertional mutagenesis (for example, if the proviral DNA randomly disrupts a tumor suppressor gene or activates an oncogene). The use of retrovirus vectors is limited by the sensitivity of the vector to inactivation, by the fact that target cells must proliferate in order to integrate the proviral DNA into the genome, and by production problems associated with recombination, rearrangements, and low titers (*1*, *2*, *5*). Retrovirus vectors have been used almost entirely in ex vivo gene transfer trials.

Adenovirus vectors in current use accommodate expression cassettes up to 7.5 kb (*1*, *2*, *6*). These vectors enter the cell by means of two receptors: a specific receptor for the adenovirus fiber and $\alpha_v\beta_3$ (or $\alpha_v\beta_5$) surface integrins that serve as a receptor for the adenovirus penton (*7*) (Fig. 2). Adenovirus vectors are well suited for in vivo transfer applications because they can be produced in high titers (up to 10^{13} viral particles/ml) and they efficiently transfer genes to nonreplicating and replicating cells (*8*). The transferred genetic information remains epischromosomal, thus avoiding the risks of permanently altering the cellular genotype or of insertional mutagenesis. However, adenovirus vectors in current use evoke nonspecific inflammation and antivector cellular immunity (*9*). These responses, together with the epischromosomal position of the expression cassette, limit the duration of expression to periods ranging from weeks to months. Thus adenovirus vectors will have to be readministered periodically to maintain their persistent expression. Although it is unlikely that

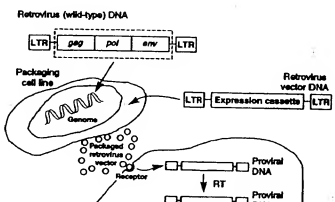

Fig. 1. Retrovirus vector design, production, and gene transfer. Retroviruses are RNA viruses that replicate through a DNA intermediate. The retrovirus vectors administered to humans all use the Maloney murine leukemia virus as the base. The *gag*, *pol*, and *env* sequences are deleted from the virus, rendering it replication-deficient. The expression cassette is inserted, and the infectious replication-deficient retrovirus is produced in a packaging cell line that contains the *gag*, *pol*, and *env* sequences that provide the proteins necessary to package the virus. The vector with its expression cassette enters the target cell via a specific receptor. In the cytoplasm, the reverse transcriptase (RT) carried by the vector converts the vector RNA into the proviral DNA that is randomly integrated into the target cell genome, where the expression cassette makes its product.

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repeat administration will be risky, it is not known whether antibodies directed against vector capsid proteins will limit the efficacy of repetitive administration of these vectors (9). Adenovirus vectors have been used only in in vivo human trials.

In theory, plasmid-liposome complexes have many advantages as gene transfer vectors, in that they can be used to transfer expression cassettes of essentially unlimited size, cannot replicate or recombine to form an infectious agent, and may evoke fewer inflammatory or immune responses because they lack proteins (10) (Fig. 3). The disadvantage of these vectors is that they are inefficient, requiring that thousands of plasmids be presented to the target cell in order to achieve successful gene transfer. The available data are not sufficient to determine if repetitive administration of liposomes or DNA poses safety risks. Plasmid-liposome complexes have been used only in in vivo human trials.

Expression cassettes and clinical targets. Human gene transfer studies fall into two categories: marking and therapeutic (Table 1). The marking studies use expression cassettes with bacterial antibiotic-resistant genes, which allow the genetically modified cells to be identified (Table 1). Because the marking genes have no function (other than to permit selection of the modified cells in vitro), the trials using marker genes have been designed to demonstrate the feasibility of human gene transfer, to uncover biologic principles relevant to human disease, and to evaluate safety. These trials have mostly used retrovirus vectors and have focused on malignant disorders or on human immunodeficiency virus (HIV) infection.

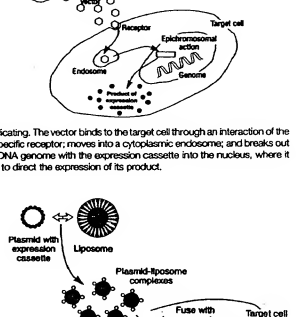

Fig. 2. Adenovirus vector design, production, and gene transfer. Adenoviruses are DNA viruses with a 36-kb genome. The wild-type adenovirus genome is divided into early (E1 to E4) and late (L1 to L5) genes. All adenovirus vectors administered to humans use adenovirus serotypes 2 or 5 as the base. The ability of the adenovirus genome to direct production of adenoviruses is dependent on sequences in E1. To produce an adenovirus vector, the E1 sequences (and E3 sequences if the space is needed) are deleted. The expression cassette is inserted, and the vector DNA is transfected into a complementing cell line with E1 sequences in its genome. The adenovirus vector with its expression cassette is E1$^-$ and thus incapable of replicating. The vector binds to the target cell through an interaction of the adenovirus fiber and penton, each to a specific receptor; moves into a cytoplasmic endosome; and breaks out and delivers its linear, double-stranded DNA genome with the expression cassette into the nucleus, where it functions in an episomal fashion to direct the expression of its product.

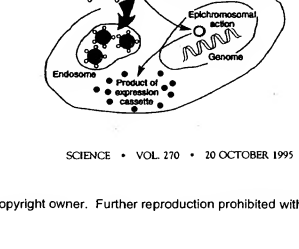

Fig. 3. Plasmid-liposome complex design and gene transfer. The liposomes used in human gene transfer trials have various compositions, but typically include synthetic cationic lipids. The positively charged liposome is complexed to the negatively charged plasmid with its expression cassette. The complexes enter the target cell by fusing with the plasma membrane. The vector does not have an inherent macromolecular structure that conveys information to enable efficient translocation of the plasmid to the nucleus. Consequently, most of the newly introduced genetic material is wasted as it is shunted to cytoplasmic organelles. When used in vivo, it is likely that most, if not all, of the plasmids that reach the nucleus function in an epischromosomal fashion.

The therapeutic trials seek to transfer expression cassettes carrying genes that will evoke biologic responses that are relevant to the treatment of human disease, and to demonstrate that this can be accomplished safely. The therapeutic studies have used retrovirus vectors, adenovirus vectors, or plasmid-liposome complexes. All of the therapeutic trials have been directed toward monogenic hereditary disorders or cancer.

What Has Really Been Accomplished?

Feasibility of gene transfer. Probably the most remarkable conclusion drawn from the human trials is that human gene transfer is indeed feasible. Although gene transfer has not been demonstrated in all recipients, most studies have shown that genes can be transferred to humans whether the strategy is ex vivo or in vivo, and that all vector types function as intended. Taken together, the evidence is overwhelming, with successful human gene transfer having been demonstrated in 28 ex vivo and 10 in vivo studies (Table 1).

In the ex vivo studies with retrovirus vectors, successful gene transfer to humans has been shown by the transfer of marker genes to various classes of T cells (11–16), to stem cells in blood and marrow (16–27), to tumor-infiltrating lymphocytes (TILs) (11, 28, 29), to neoplastic cells of hematopoietic lineage (16, 17, 20, 21, 25, 26), and to neoplastic cells derived from solid tumors (Table 1). Although there is variation among ex vivo clinical trials in the proportion of genetically marked cells recovered from the recipients, retroviral vector DNA or marker gene–derived mRNA or both have been observed in cells collected after periods ranging from several weeks to 36 months after administration.

Retrovirus vectors also have been used to transfer therapeutic genes ex vivo, with success demonstrated by the fact that the modified cells exhibit their altered phenotype in vivo for up to 36 months (Table 1). Typically, the expression cassette containing the therapeutic gene also contains an antibiotic-resistance gene, permitting the ex vivo selection of genetically modified cells recovered from the recipient. Successful gene transfer has been demonstrated in cells recovered from children with adenosine deaminase (ADA) deficiency after transfer of the normal ADA complementary DNA (cDNA) to autologous T cells, cord blood, and placental cells (30–32); from individuals with solid tumors after transfer of cytokine cDNAs in autologous vaccine strategies to fibroblasts, TILs, or tumor cells (33–37); from individuals with familial hypercholesterolemia after transfer of the low-density lipoprotein (LDL) receptor cDNA to autologous hepatocytes (38, 39);

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from HIV+ siblings after transfer of a chimeric T cell receptor cDNA to blood T cells of a twin (*40*); and from individuals with tumors who received autologous marrow transplants after transfer of the multidrug resistance 1 cDNA to autologous blood CD34+ stem cells (*41*). A retrovirus vector has also been used in vivo to successfully transfer a p53 antisense cDNA in lung carcinoma cells (*42*). Finally, in a combined ex vivo–in vivo strategy for treatment of brain neoplasms, gene transfer to tumor cells has been observed after xenogeneic cells (murine fibroblasts whose genome had been modified with amphotropic packaging sequences) infected with a retrovirus vector containing an expression cassette with the herpes simplex thymidine kinase (HSTK) gene were introduced into the tumor (*43*).

In in vivo studies with adenovirus vectors, several studies have shown that direct administration of a vector containing the normal human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the nasal or bronchial epithelium of individuals with cystic fibrosis (CF) results in transfer of the CFTR cDNA-containing expression cassette to the epithelium, where CFTR mRNA or protein is expressed for at least 9 days (*44–50*) (Table 1). Direct administration of a plasmid-liposome complex containing an expression cassette with the CFTR cDNA to the nasal epithelium of individuals with CF resulted in expression of CFTR mRNA in the epithelium (*51*). Finally, plasmid-liposome complexes have

Table 1. Summary of studies showing that transfer of genes to humans is feasible. Data shown are based on published articles and abstracts and on RAC-mandated biannual reports of principal investigators as of the RAC meeting of 8 to 9 June 1995. Abbreviations used for vector study type are RV, retrovirus; Ad, adenovirus; PL, plasmid-liposome complex; M, marker-type study; and T, therapeutic-type study. Abbreviations used for gene products are Neo^R, neomycin phosphotransferase; Hygro, hygromycin phosphotransferase; HSTK, herpes simplex thymidine kinase; ADA, adenosine deaminase; LDLR, low-density lipoprotein receptor; TNF, tumor necrosis factor α; CD4 zeta-R, chimeric T cell receptor; MDR-1, multidrug resistance 1; IL-4, interleukin 4; GM-CSF, granulocyte macrophage colony-stimulating factor; CFTR, cystic fibrosis transmembrane conductance regulator; and B7 + β_2, histocompatibility locus antigen class I–B7 + β_2 microglobulin. Except for Neo^R, Hygro, and HSTK, all genes are cDNAs. Abbreviations used for target cells are TIL, tumor-infiltrating lymphocytes; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus 1; and CTL, cytotoxic T lymphocytes. All target cells are autologous unless otherwise specified. Abbreviations used to characterize study populations are AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; ca, carcinoma; and CF, cystic fibrosis. Under in vivo evidence of gene transfer, a plus sign indicates a report of transfer or expression (or both) of an exogenous gene in cells obtained from one or more individuals in the study; time listed is the longest time after administration that gene transfer or expression was observed.

Vector study type	Gene product	Target cells	Study population	In vivo evidence of gene transfer	Principal investigator	Reference number
RV-M	Neo^R‖	TIL	Melanoma	+ 2 months	Rosenberg, S. A.	(*26*)
RV-M	Neo^R‖	TIL	Melanoma	+ 3 months	Lotze, M. T.	(*29*)
RV-M	Neo^R‖	Marrow	AML	+36 months	Brenner, M. K.	(*16, 17*)
RV-M	Neo^R‖	Marrow	Neuroblastoma	+29 months	Brenner, M. K.	(*18*)
RV-M	Neo^R‖	Marrow	Neuroblastoma	+20 months	Brenner, M. K.	(*18*)
RV-M	Neo^R‖	Marrow	CML	+ 5 months	Deisseroth, A. B.	(*20*)
RV-M	Neo^R‖	Marrow	AML, ALL	+12 months	Cornetta, K.	(*21*)
RV-M	Neo^R‖	CD4+, CD8+; blood, TIL	Melanoma, renal cell ca	+	Economou, J. S.	(*11*)
RV-M	Neo^R‖	CD34+ blood, marrow	Multiple myeloma	+18 months	Dunbar, C. E.	(*22, 23*)
RV-M	Neo^R‖	CD34+ blood, marrow	Breast ca	+18 months	Dunbar, C. E.	(*23, 24*)
RV-M	Neo^R‖	Marrow	AML	+12 months	Brenner, M. K.	(*25*)
RV-M	Neo^R‖	Normal twin blood T cells†	Identical twins, 1 HIV+	+ 4 months	Walker, R. E.	(*12*)
RV-M	Neo^R‖	Blood, marrow	CML	+	Deisseroth, A. B.	(*26*)
RV-M	Neo^R‖	CD34+ blood	Metastatic ca, lymphoma	+15 days	Schuening, F. G.	(*27*)
RV-M	Neo^R‖	EBV-specific CTL‡	Ca, leukemia	+ 7 months	Heslop, H. E.	(*14, 15*)
RV-M	Hygro + HSTK	CD8+ HIV *gag* specific, CTL§	HIV+, lymphoma	+14 days	Greenberg, P.	(*13*)
RV-T	ADA	Blood T cells	ADA deficiency	+36 months	Blaese, R. M.	(*30, 31*)
RV-T	ADA	Cord blood cells	ADA deficiency	+18 months	Blaese, R. M.	(*30, 32*)
RV-T	LDLR	Hepatocytes	Familial hypercholesterolemia	+ 4 months	Wilson, J. M.	(*38, 39*)
RV-T	TNF	TIL	Melanoma	+	Rosenberg, S. A.	(*33*)
RV-T	IL-2	Tumor cells‖	Metastatic ca	+	Rosenberg, S. A.	(*36*)
RV-T	IL-2	Neuroblastoma‖	Metastatic ca	+	Brenner, M. K.	(*35*)
RV-T	CD4 zeta-R	Normal twin blood T cells†	Identical twins, 1 HIV+	+ 4 months	Walker, R. E.	(*40*)
RV-T	MDR-1	Blood CD34+	Breast ca	+	Deisseroth, A. B.	(*41*)
RV-T	IL-4	Fibroblasts¶	Metastatic ca	+	Lotze, M. T.	(*34*)
RV-T	GM-CSF	Melanoma‖	Melanoma	+	Dranoff, G.	(*37*)
RV-T	Anti-sense p53	Lung ca	Lung ca	+ 1 days	Roth, J. A.	(*42*)
RV-T*	HSTK	Tumor cells	Glioblastoma	+	Oldfield, E. H.	(*43*)
Ad-T	CFTR	Nasal, airway epithelium	CF	+ 9 days#	Crystal, R. G.	(*44, 45*)
Ad-T	CFTR	Nasal epithelium	CF	+	Welsh, M. J.	(*46, 47*)
Ad-T	CFTR	Nasal epithelium	CF	+	Welsh, M. J.	(*48*)
Ad-T	CFTR	Airway epithelium	CF	+ 5 days**	Wilson, J. M.	(*49*)
Ad-T	CFTR	Nasal epithelium	CF	+	Boucher, R. C.	(*50*)
PL-T	CFTR	Nasal epithelium	CF	+ 4 days	Geddes, D. M.	(*51*)
PL-T	B7 + β_2	Melanoma	Metastatic ca	+ 3 days	Nabel, G. J.	(*52*)
PL-T	B7 + β_2	Colorectal ca	Metastatic ca	+	Rubin, J. T.††	(*53, 54*)
PL-T	B7 + β_2	Renal cell ca	Metastatic ca	+	Volgelzang, N.††	(*54, 55*)
PL-T	B7 + β_2	Melanoma	Metastatic ca	+	Hersh, E.††	(*56*)

*This study used a mixed ex vivo–in vivo strategy, in which a xenogenic fibroblast cell line was modified with a retrovirus to produce an amphotropic retrovirus vector containing an expression cassette with the genes for Neo^R + HSTK, and the modified retrovirus-producing cell line was administered directly into the tumor. †Blood T cells from a normal identical twin modified with an expression cassette and then administered to an HIV+ twin. ‡Allogeneic. §The HSTK gene used as a marker gene. ‖Autologous tumor cells modified with an expression cassette, lethally irradiated, and then administered as a "vaccine." ¶Autologous fibroblasts modified with an expression cassette, lethally irradiated, and then administered together with autologous, unmodified tumor cells as a "vaccine." #Messenger RNA at 9 days, vector DNA at 15 days. **A few + cells were observed at 90 days. ††Collaborative study, different institutions.

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been used to transfer the human leukocyte antigen (HLA)–B7 and β2 microglobulin cDNAs directly to solid tumors in vivo, with consequent expression of the transfer cassette being seen in the tumor (*52–56*).

Relevant biologic responses. No human disease has been cured by human gene transfer, and it is not clear when this will be accomplished. However, several studies have demonstrated that therapeutic genes transferred to humans by means of retrovirus, adenovirus, and plasmid-liposome vectors can evoke biologic responses that are relevant to the gene product and to the specific disease state of the recipient (Table 2). Most of the studies demonstrating biologic efficacy have focused on monogenic hereditary disorders, where it is rational to believe that, if the normal gene product could be appropriately expressed at the relevant site, the abnormal biologic phenotype could be corrected.

Severe combined immunodeficiency–ADA deficiency is a fatal recessive disorder caused by mutations in the gene encoding ADA; these mutations cause accumulation of adenosine and 2′-deoxyadenosine, which are toxic to lymphocytes (*57*). Affected children are unable to generate normal immune responses and develop life-threatening infections. The normal ADA cDNA was transferred ex vivo with a retrovirus vector into T lymphocytes of two children with this disorder, and the modified T cells were expanded in the laboratory and periodically infused into the autologous recipients (*30, 31*). This resulted in an increase in T cell numbers and in the ADA levels in circulating T cells. The two children now have partially reconstituted immune function, as demonstrated by T cell cytokine release, cytotoxic T cell activity, isohemagglutinin production, and skin test responses to common antigens. In addition, three infants with ADA deficiency who received autologous infusions of cord blood $CD34^+$ stem cells modified ex vivo with a retrovirus vector containing the normal ADA cDNA have also shown evidence of increased numbers of blood T cells and increased ADA levels in T cells (*30, 32*). The results of the ADA studies are difficult to interpret, because none of these trials have been controlled and the recipients have also received the standard therapy of enzyme infusions with mono-methoxypolyethylene glycol-bovine ADA. Despite these caveats, these observations are consistent with the conclusion that this ex vivo gene transfer strategy evokes biologic responses that are relevant to treatment of ADA deficiency.

Familial hypercholesterolemia is a fatal disorder caused by a deficiency of LDL receptors in the liver that are secondary to mutations in the LDL receptor genes (*38, 39, 58*). The consequences are high levels of serum cholesterol and LDL cholesterol, premature atherosclerosis, and myocardial infarction. A retrovirus vector was used ex vivo to transfer the normal LDL receptor cDNA to autologous hepatocytes obtained by partial liver resection of an individual with this disorder (*38, 39*). After reinfusion of the modified hepatocytes into the liver via the portal vein, there was a reduction in LDL cholesterol and in the ratio of LDL to high-density lipoprotein over 18 months, which is consistent with the concept that the corrected cells functioned in vivo to internalize and metabolize LDL cholesterol appropriately. Like the ADA deficiency studies, this study was partially compromised because other therapies were being administered. Furthermore, the LDL receptor gene mutations were mild and could have responded to experimental variables other than the transferred gene (*58*). However, similar transfer of autologous hepatocytes modified ex vivo to other individuals with more severe mutations of the LDL receptor gene demonstrated partial correction of a variety of lipoprotein-related metabolic parameters, which is consistent with the conclusion that this gene transfer strategy did evoke a relevant response (*38*).

Cystic fibrosis is the most common lethal hereditary disorder in North America (*59*). It is caused by mutations in the CFTR gene, a gene coding for an adenosine 3′,5′-monophosphate (cAMP)–regulatable chloride channel in the apical epithelium. As a result of these mutations, the airway epithelium is deficient in CFTR function. This leads to chronic airway infection and inflammation and progressive respiratory derangement. There is compelling logic to the argument that these lung derangements could be prevented if CFTR function could be restored in these cells (*60*). It is difficult to assess CFTR function in the airway epithelium in vivo in humans, but the nasal

Table 2. Data from human gene transfer studies in which transfer of genetic material has evoked a biologic response that is relevant to the underlying disease.

Disease category	Disease	Strategy	Vector	Gene product*	Target cells	Relevant biologic response	Principal investigator	Reference number
Hereditary	ADA deficiency	Ex vivo	Retrovirus	ADA	Blood T cells and cord blood $CD34^+$ stem cells	Partial restoration of immune response	Blaese, R. M.	(*30–32*)
	Familial hypercholesterolemia	Ex vivo	Retrovirus	LDLR	Hepatocytes	Partial correction of lipid abnormalities	Wilson, J. M.	(*38, 39*)
	Cystic fibrosis	In vivo	Adenovirus	CFTR	Nasal epithelium	Partial correction of potential difference abnormalities across the nasal epithelium	Welsh, M. J. Crystal, R. G.	(*46, 47*) (*44, 62*)
	Cystic fibrosis	In vivo	Plasmid-liposome complex	CFTR	Nasal epithelium	Partial correction of potential difference abnormalities across the nasal epithelium	Geddes, D. M.	(*51*)
Acquired	Solid tumors	In vivo	Plasmid-liposome complex	HLA-B7 + β_2	Tumor cells†	Specific immune response to tumor	Nable, G. J. Rubin, J. Vogelzang, N. Hersh, E.	(*52*) (*53, 54*) (*54, 55*) (*54, 56*)
		Ex vivo	Retrovirus	IL-4	Fibroblasts‡§	Specific and nonspecific immune response to tumor	Lotze, M.	(*34*)
		Ex vivo	Retrovirus	IL-2	Neuroblastoma‡	Specific and nonspecific immune response to tumor	Brenner, M. K.	(*35*)

*ADA, adenosine deaminase deficiency; LDLR, low-density lipoprotein receptor; CFTR, cystic fibrosis transmembrane conductance regulator; HLA-B7 + β_2, histocompatibility locus antigen class I-B7 + β_2 microglobulin; IL-4, interleukin-4. †Direct administration to melanoma, colorectal carcinoma, or renal cell carcinoma. ‡Lethally irradiated, used as a "vaccine." §Combined with lethally irradiated, unmodified autologous tumor cells.

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epithelium has been used as a surrogate to test the hypothesis that in vivo transfer of the normal CFTR cDNA will correct the functional consequences of CFTR deficiency (*47, 61*). The parameters measured relate to the observation that the deficiency in CFTR causes an abnormal potential difference between the nasal epithelial surface and subcutaneous tissues. Although the nasal epithelium is not identical to the airway epithelium, two of three studies with adenovirus vectors (*44–47, 50, 62*) and one with plasmid-liposome complexes (*51*) have demonstrated that in vivo transfer of the CFTR cDNA to the nasal epithelium evokes a partial correction of these potential difference abnormalities for 1 to 2 weeks.

There are also studies in which human gene transfer appears to have initiated biologic responses that are relevant to therapy for an acquired disorder. These are all "tumor vaccine" studies, based on the hypothesis that exaggerated local expression of an immune-related cytokine might help activate the immune system sufficiently to recognize tumor antigens and control the growth of tumor cells. In one ex vivo study, a retrovirus vector was used to transfer the interleukin-4 (IL-4) cDNA to autologous fibroblasts (*34*). The cells were then irradiated and implanted subcutaneously in the donor together with irradiated, unmodified, autologous tumor cells. In some recipients, this evoked infiltration with $CD3^+$ T cells and tumor-specific $CD4^+$ T cells at the immunization site, as well as enhanced expression of cell adhesion molecules on capillary endothelium. In another trial, autologous neuroblastoma cells modified ex vivo with a retrovirus to contain the IL-2 cDNA were lethally irradiated and implanted subcutaneously (*35*). In some individuals, this evoked systemic augmentation of $CD16^+$ natural killer cells and tumor-specific $CD8^+$ cytotoxic T cells and eosinophilia. Finally, in four trials, in vivo plasmid-liposome complexes were used to transfer a heterologous HLA class I–B7 cDNA and the β_2 microglobulin cDNA directly to solid tumors (*52–56*). In several patients, there was evidence that the gene transfer process initiated amplification of the numbers of detectable, circulating, tumor-specific cytotoxic T cells.

Insights into human biology. Experience with marking studies has shown that human gene transfer can yield important insights into human biology by making it possible to track the fate of genetically marked cells in a recipient. For example, when stored autologous marrow is used to rescue a patient from the suppression of marrow function that complicates high-dose chemotherapy for late-stage malignancy, the individual may subsequently develop a recurrence of the malignancy. Gene transfer marking studies have helped answer the question of whether the recurrence is secondary to a residual tumor in the patient or is derived from malignant cells contaminating the reinfused banked marrow. Several studies that used an ex vivo strategy with a retrovirus vector to mark marrow cells with a neomycin resistance (neo^R) gene and then reinfused the marked marrow have demonstrated that contamination of the autologous marrow with malignant cells is common (*11, 16–25*). These observations have led to more attention being focused on purging banked autologous marrow of contaminating neoplastic cells before they are reinfused.

There are a number of strategies being developed for the use of ex vivo gene transfer to protect autologous T cells from infection with the HIV-1. None will work, however, if autologous T cells manipulated in the laboratory and then reinfused into an HIV^+ individual have a short biologic half-life. The life-span of an autologous T cell in HIV^+ individuals has been evaluated in identical twin pairs in which one twin is HIV^+ and the other is HIV^- (*12*). A retrovirus vector was used ex vivo to transfer the neo^R gene into the T cells from the normal twin, and the genetically marked cells were then reinfused into the HIV^+ twin. Some $CD4^+$ and $CD8^+$ marked T cells (or their progeny) survived for at least 10 months, providing a baseline to allow future studies to compare the fate of T cells that have been genetically modified to prevent HIV infection.

In a strategy to prevent reactivation of Epstein-Barr virus (EBV) and the accompanying associated lymphoproliferative disease after bone marrow transplantation, allogenic EBV-specific cytotoxic T cells (CTL) were genetically marked with a retrovirus vector, and the cells were infused into individuals at risk (*15, 16*). This preliminary study suggested that EBV-specific allogenic cells may help control EBV-associated complications of marrow transplantation, and the use of the marker genes demonstrated that the infused EBV-specific CTL persisted in the recipients for 10 weeks.

Two types of therapeutic studies support the biologic concept that minimal correction of a genotype can have significant phenotypic consequences. In the ex vivo study of retrovirus-mediated transfer of the LDL receptor cDNA into autologous hepatocytes in patients with familial hypercholesterolemia, liver biopsy several months after reinfusion of the modified hepatocytes showed that at most 5% of the total hepatocyte population expressed the normal gene in vivo (*38, 39, 62*). Despite this minimal correction, in some of the recipients there were changes in LDL-related parameters that suggested LDL receptor function in the liver had been partially restored. Partial phenotypic correction has also been observed in most of the trials of adenovirus- and plasmid-liposome complex–mediated in vivo transfer of the CFTR cDNA to the nasal epithelium in CF, even though the amount of gene transfer and expression has been limited to a small fraction of the target cells (*44–47, 50, 51, 62*).

Finally, when adenovirus vectors are administered to experimental animals, the animals quickly develop circulating neutralizing antibodies directed against the vector (*9*). In two studies of administration of adenovirus vectors to the airways of individuals with CF, no circulating neutralizing antibodies were detected (*44, 45, 49*). This is an important observation, because the expression cassette delivered by adenovirus vectors remains epischromosomal, and thus the vector will have to be readministered as its expression wanes. Although it is possible that there are local antibodies to the vectors in these individuals (*9*), the lack of a systemic immune response to such an antigen load is encouraging in that it suggests that antibodies to vectors may not be a major factor limiting persistent vector expression in humans when the lung is repeatedly dosed (*64*).

Safety of gene transfer. The theoretical safety concerns regarding human gene transfer are not trivial. For the individual recipient, there is the possibility of vector-induced inflammation and immune responses, of complementation of replication-deficient vectors leading to overwhelming viral infection, and (for the retrovirus vectors) of insertional mutagenesis. There are also theoretical issues that are important to society, including concerns about modifying the human germ line and about protecting the environment from new infectious agents generated from gene transfer vectors carrying expression cassettes with powerful biologic functions.

There have been adverse events in the human gene transfer trials, including inflammation induced by airway administration of adenovirus vectors (*44–50, 65*) and by administration to the central nervous system of a xenogenic producer cell line releasing a retrovirus vector (*43, 66*). However, compared with the total numbers of individuals undergoing gene transfer, adverse events have been rare and have been related mostly to the dose and the manner in which the vectors were administered. Shedding of viral vectors in the in vivo trials was very uncommon and was limited in extent and time (*42, 44–50, 65*). No novel infectious agents generated from recombination of the transferred genome and the host genome or other genetic information have been detected, not has any replication-competent virus related to the vector. Cells modified ex vivo with retrovirus vectors have been infused repeti-

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tively without adverse effects (13, 30, 31, 35), adenovirus vectors have been administered repetitively in vivo to the nasal (48) and bronchial epithelium safely (64, 67), and plasmid-liposome complexes have been administered repetitively to tumors in vivo without complications (52–56). Finally, human gene transfer has not been implicated in initiating malignancy, although the numbers of recipients and time of observation will have to be much greater to allow definitive conclusions regarding this issue.

What Are the Obstacles to Successful Human Gene Transfer?

With the successes of the human gene transfer trials have come the sobering realities of the drug development process. Some of the problems are generic for the field, and some are specific for each vector.

Inconsistent results. All of the human gene transfer studies have been plagued by inconsistent results, the bases of which are unclear. For example, in the two children with ADA deficiency receiving intermittent infusions of autologous T cells modified ex vivo with the normal ADA cDNA, the resulting proportion of ADA^+ circulating T cells has varied from 0.1 to 60% (30, 31). In the CF trials, there is evidence that adenovirus vectors and plasmid-liposome complexes can transfer the normal CFTR cDNA to the respiratory epithelium, but expression is observed in at most 5% of the target cells and is not seen in all recipients (44–51, 65). Further, an appropriate biologic response to gene transfer (correction of the abnormal potential difference across the nasal epithelium) has been observed in some patients in most, but not all, of the studies of CFTR cDNA transfer (44–47, 50, 51, 62). In most of the ex vivo marrow-marking trials, successful gene transfer is observed intermittently (Table 1).

Humans are not simply large mice. There have been several surprise examples, in which predictions from gene transfer studies in experimental animals have not been borne out in human safety and efficacy trials. In tumor vaccine studies intended to evoke a tumor-directed immune response, there is no convincing evidence (other than anecdotal case reports) that tumors regress, despite the promising observations in experimental animals (34, 37, 52–56). It has also become apparent that studies in experimental animals may not necessarily predict the toxicology of vectors in humans. In one patient with CF in whom 2×10^9 plaque-forming units of an adenovirus vector containing the CFTR cDNA were administered to the lung, a transient local and systemic inflammatory syndrome was evoked, despite the fact that no clinically apparent toxicity was observed in rodents and nonhuman primates receiving 1000-fold greater doses by the same route (45). Likewise, in an ex vivo–in vivo strategy to treat glioblastoma, transfer of xenogenic retrovirus-producing cells to the tumor was accomplished without significant adverse effects in experimental animals, but the human studies have been associated with central nervous system toxicity related to transfer of the cell line to the tumor (43, 66).

Production problems. There are significant hurdles in vector production that must be overcome before large clinical trials can be initiated. Generation of replication-competent virus is observed in production of clinical-grade retrovirus and adenovirus vectors; and lack of reproducibility, aggregation, and contamination with endotoxin complicate the production of clinical-grade plasmid-liposome complexes (68).

The perfect vector. The ideal gene transfer vector would be capable of efficiently delivering an expression cassette carrying one or more genes of the size needed for the clinical application. The vector would be specific for its target, not recognized by the immune system, stable and easy to reproducibly produce, and could be purified in large quantities at high concentrations. It would not induce inflammation and would be safe for the recipient and the environment. Finally, it would express the gene (or genes) it carries for as long as required in an appropriately regulated fashion (69).

This ideal vector is conceptually impractical, because the human applications of gene transfer are broad, and the ideal vector will likely be different for each application. Clinical experience to date suggests that retrovirus, adenovirus, and plasmid-liposome vectors all need refinement, but each is relatively well suited for the clinical targets at which they have been directed. Further, the technology is now available to create designer vectors that can be optimized for each application. Among the design hurdles for all vectors are the need to increase the efficiency of gene transfer, to increase target specificity and to enable the transferred gene to be regulated. Reproducible production of large amounts of pure vector is a hurdle for all classes of vectors. Some of the vector-specific hurdles are reduction of the risk for insertional mutagenesis in retrovirus vectors, minimization of the amount of immunity and inflammation evoked by the adenovirus vectors, and enhancement of the translocation of the gene to the nucleus for the plasmid-liposome complexes.

There is considerable interest in developing new vectors, but there is controversy as to which vector class is most likely to succeed, particularly for use in in vivo applications. There are two philosophical camps in vector design: viral and nonviral. The viral proponents believe that the most efficient means to deliver an expression cassette in vivo is to package it in a replication-deficient recombinant virus. The logic supporting this approach is the knowledge that viruses are masterful at reproducing themselves, and thus have evolved strategies to efficiently express their genetic information in the cells they infect. The nonviral proponents concede this argument but believe that the redundant anti-immune and inflammatory host defenses against viruses may be a risk to recipients, will limit the duration of expression as the infected cells are recognized by the immune system, and may hinder the efficacy of repeat administration of the vectors. Thus, nonviral vector aficionados believe it is rational to start from scratch to design safe, efficient, gene transfer strategies. In contrast, the viral camp believes that it is best to start with something that works but then to circumvent the replication, immune, and inflammation risks inherent in their use by appropriate vector design. It is most likely that these philosophical differences will eventually disappear as new classes of vectors are designed that incorporate features of viral and nonviral vectors, as dictated by specific clinical applications.

Future Prospects

None of the drug development problems facing human gene transfer are insurmountable, but each will take time to solve. However, the logic underlying the potential usefulness of human gene transfer is compelling; and put in a context in which the human genome project will provide 80,000 to 100,000 human genes that could be used in expression cassettes for human gene transfer, the potential impact of this technology for innovative therapies and increased understanding of human biology is enormous.

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Gene Therapy for Cancer

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WHY GENE THERAPY?

It is well established that most cancers result from a series of accumulated, acquired genetic lesions in somatic cells that are faithfully reproduced until a malignant clone is created, which is ultimately able to destroy the host. To a larger and larger extent, the genetic lesions associated with malignant transformation and progression in a wide variety of human cancers are being identified [1, 2]. Armed with this knowledge of the molecular anatomy of the cancer cell, gene therapy has emerged as a new method of therapeutic and possibly preventive intervention against cancer targeted at the level of cellular gene expression [3]. In this approach, the complex cancerous pathophysiological state is altered by delivering nucleic acids into tumoral or normal cells. These nucleic acids may be genes, portions of genes, oligonucleotides, or RNA. In conventional therapeutics, as in pharmacotherapy, a cell or tissue phenotype is altered by modifying cell physiology or metabolism at the level of protein expression. In contrast, in gene therapy this is accomplished by changing the pattern of expression of genes whose products may thus achieve the desired effect on the cellular phenotype.

In the treatment of human disease, gene therapy strategies may offer the potential to achieve a much higher level of specificity of action than conventional drug therapeutics by virtue of the highly specific control and regulatory mechanisms of gene expression that may be targeted. Additionally, interceding at an earlier, upstream step in disease pathogenesis may offer greater potential to induce fundamental changes in phenotypic parameters of disease, with a more favourable clinical outcome. The availability of gene transfer systems, or vectors, for permanent or long-term genetic modification of cells and tissues should allow definitive therapeutic or preventive interventions. Furthermore, gene transfer may be accomplished in a limited loco-regional context, producing a high concentration of therapeutic molecules in the local area. Thus, undesired systemic effects of those therapeutic molecules are avoided. Lastly, using the body to produce therapeutic proteins, potentially in only certain tissues, has practical advantages of its own [4]. Briefly, limitations associated with manufacture, stability, and duration of effect after administration of drugs based on synthetic peptides are completely avoided. From the same pharmacological point of view, designer drugs based on small molecules, currently under intensive investigation, can hardly substitute the function of complex defective proteins, such as many products of tumour suppressor genes.

In the treatment of human malignant tumours, several obstacles explain the limitations of currently available treatments for achieving definitive cures in most cases of advanced disease (Table 1). It is apparent that a combination of new chemotherapy drugs, higher doses of drugs, novel cytokines, improved regimens of radiotherapy, and more sophisticated surgery can achieve incremental improvements in cancer treatment. But these therapies do not address critical biological obstacles and, thus, probably will not bring the much-needed radical advances in the implementation and results of cancer treatment. In contrast, gene therapy offers the potential for overcoming some of these fundamental barriers (Table 1).

Table 1. Potential contributions of gene therapy to overcome obstacles for curing cancer

	Obstacles to curing cancer	Potential contribution of gene transfer
1	All tumours are *genetically unstable* and thus they are extraordinarily adaptable to environmental changes	Gene transfer of DNA repair or cell cycle checkpoint genes that restore DNA stability and cell susceptibility to therapeutic insults
2	Tumours are heterogeneous in many respects, including genetic mutations, expression of oncoproteins, immunogenicity, response to environmental changes, etc.	Targeting of genetically homogeneous and stable tissues, such as the tumour vasculature and stroma; genetic immunopotentiation; chimeric vectors
3	As a consequence of obstacles 1 and 2, tumours have, or acquire, *resistance* to cellular toxins and to many other therapeutically induced cellular insults	Strategies above, associated with chemotherapy or radiotherapy or with the transfer of additional genes that sensitise tumour cells to drugs or radiation
4	Tumours can have a low *cellular growth fraction*; therefore, they are less susceptible to mitotic toxins and to gene transfer vectors that require dividing cells	Use of vectors that do not require cellular division for gene delivery and expression (adenovirus, herpesvirus, lentivirus, chimeric vectors), repeated administration of non-immunogenic vectors
5	Tumours form *metastases*, which have to be reached systemically to eradicate the tumour completely	Use of targetable, injectable vectors (tropism-modified viruses, cellular vehicles, liposomes); genetic immunopotentiation
6	Tumours do not express specific tumour antigens or immune costimulatory molecules; alternatively, tumours down-regulate antigen presentation, induce *immunological tolerance*, or inhibit the effector mechanisms of the immune response	Transfer of genes encoding costimulatory molecules and cytokines; genetic modification of antigen-presenting cells; induce inflammatory reactions that activate antigen presentation; transfer of genes blocking tumour-secreted inhibitors of the immune response
7	The *spontaneous* behaviour of human tumours is somewhat different from that of malignant cells *in vitro*, and from that of experimental tumours in animal models	Development of better animal models, including tumour models in transgenic mice
8	Tumours are diagnosed in *advanced stages*, when billions of tumour cells exist in the body, frequently widely disseminated	Development of amplification vector systems (replicative viral vectors and exploitation of bystander effects); use of targetable, injectable vectors; genetic immunopotentiation
9	The understanding and treatment of cancer requires the contribution of very *diverse fields* of basic knowledge, biotechnology, and medical practice	*De facto* multidisciplinary recruitment of gene therapy researchers

STRATEGIES

A number of strategies have been developed to accomplish cancer gene therapy. These approaches include (1) mutation compensation, (2) molecular chemotherapy, and (3) genetic immunopotentiation. For mutation compensation, gene therapy techniques are designed to correct the molecular lesions that are aetiologic of malignant transformation, or avoid the contribution to malignant growth by tumour-supporting non-malignant stromal cells. For molecular chemotherapy, methods have been developed to achieve selective delivery or expression of a toxin gene in cancer or stromal cells to induce their eradication, or alternatively to increase their sensitivity to concomitant chemotherapy or radiotherapy. Also, attempts have been made to deliver genetic sequences that protect normal bone marrow cells from the toxic effects of standard chemotherapeutic drugs, thus allowing the administration of higher drug doses without reaching otherwise limiting myelosuppression. Genetic immunopotentiation strategies attempt to achieve active immunisation against tumour-associated antigens by gene transfer methodologies. Both tumour cells and cellular components of the immune system have been genetically modified to this end. Importantly, each of these approaches has been rapidly translated into human gene therapy clinical trials [5] as summarised in Table 2.

In this review, we examine the lessons learned from the results of the first attempts to apply each gene therapy strategy in human cancers. In each section, we show both the rationale of gene therapy and the problems encountered in its development, emphasising the general biological concepts of each therapeutic strategy. Finally, we illustrate prospects for overcoming the obstacles to implementation of gene therapy by novel methods that are currently being refined.

MUTATION COMPENSATION

The knowledge of the major role that growth factors, signalling molecules, cell cycle regulators, and determinant factors of angiogenesis, invasiveness and metastasis play in neoplastic progression has positive implications for gene therapy. That is, it is possible to abrogate the malignant phenotype by correcting the underexpression of tumour suppressor genes or overexpression of oncogenes involved in these phenomena. At the level of the single cell, the inactivation of tumour suppressor genes contributes to the neoplastic phenotype by abrogating critical cell cycle checkpoints, DNA repair mechanisms, and pro-apoptotic controls. To approach this loss of function, the logical intervention is replacement of the deficient function with the wild-type gene. Mutations of more than 24 tumour suppressor genes have been described in numerous cancers. Of these, *p53*, *RB1*, and *BRCA1* are currently being administered in clinical trials as replacements for the mutated counterparts (Table 3). In all these cases, pre-clinical studies showed some expression of the wild-type protein after gene delivery and reversion of the malignant phenotype, frequently associated with the induction of apoptosis in tumour cells [6]. Of note, however, some tumours have shown persistent tumorigenicity and proliferation after successful restoration and expression of wild-type genes, a phenomenon referred to as 'tumour suppressor resistance'. Other major obstacles are mentioned later.

For dominant oncogenes, it is the aberrant expression of the corresponding gene product that elicits the associated neoplastic transformation. In this context, the molecular therapeutic intervention is designed to ablate expression of the dominant oncogene. Inhibition of oncogenic function can be attempted at three levels. First, transcription of the oncogene can be inhibited. This approach uses triplex-forming oligonucleotides or other sequences that bind transcriptional start sites in the genomic DNA. An example, currently being clinically tested, is based on the adenoviral gene E1A, that inhibits transcription of the human *c-erbB-2* promoter and accordingly suppresses the tumorigenicity and metastatic potential induced by the *erbB-2* oncogene. Second, translation of the oncogene messenger RNA can be blocked using specific antisense sequences, which function by promoting degradation of the complementary message [7]. Evidence for a specific effect of antisense molecules has been particularly compelling in selected cases, and these molecules are currently undergoing clinical tests. These include antisense sequences against insulin-like growth factor 1 in glioma, K-*ras* in lung cancer, c-*myc* in breast and prostate cancer, and TGFβ in glioma (Table 3). Practical constraints have limited wide employment of this technology in protocols of human anticancer gene therapy, including the idiosyncratic efficacy of specific antisense for a given target gene and the suboptimal delivery of antisense molecules. Third, mobilisation of the nascent oncoprotein can be blocked or its function can be inhibited when in its final cell location. These last strategies involve the use of 'intracellular antibodies' that intercept and interfere with the intracellular processing of the oncoprotein, or the heterologous expression of mutant proteins that can inhibit the function of the native oncoprotein,

Table 2. Clinical trials of gene therapy for the treatment of cancer

Strategy	Clinical trials*	Molecular mechanism of anticancer effect
Mutation compensation	6	Inhibition of expression of dominant oncogenes.
	18	Augmentation of deficient tumour suppressor genes.
	2	Abrogation of autocrine growth factor loops (single chain antibodies)
Molecular chemotherapy	27	Selective delivery of toxin or toxin gene to cancer cells.
	9	Chemoprotection of normal tissues during high-dose chemotherapy.
Genetic immunopotentiation	54	*In vitro* transduction—augmentation of tropism or cell killing capacity of tumour-infiltrating lymphocytes; genetic modification of irradiated tumour cells.
	47	*In vivo* transduction—administration of costimulatory molecules or cytokines; immunisation with virus encoding tumour-associated antigens.
Viral-mediated oncolysis	2	Tumour cell lysis by viral vector replication.

*Registered in the NIH Office of Recombinant DNA Activities in December 1998 (http://www.nih.gov/od/orda/protocol.pdf)

Table 3. Mutations compensation strategies used clinically

Target*	Strategy	Vector	Tumour type
p53	Replacement of tumour suppressor gene	Adenovirus	Non-small cell lung cancer, head and neck squamous cell carcinoma, hepatic metastases of colon cancer, hepatocellular carcinoma, prostate cancer, breast cancer.
RB (Retinoblastoma)	Replacement of tumour suppressor genes	Adenovirus	Bladder cancer
BRCA-1	Replacement of tumour suppressor gene	Retrovirus	Ovarian cancer
erbB-2	Inhibition of promoter by E1A	Cationic liposome complex	Breast and ovarian cancers overexpressing erbB-2
Insulin-like growth factor 1	Blockade by antisense	Cationic liposome complex	Glioblastomas
k-ras	Blockade by antisense	Retrovirus	Non-small cell lung cancer
c-myc, c-fos	Blockade by antisense	Retrovirus	Breast and prostate cancers
TGFβ	Blockade by antisense	Plasmid and electroporation	Glioblastoma
erbB-2	scFv	Adenovirus	Ovarian cancer

*Registered in the NIH Office of Recombinant DNA Activities. December of 1998 (http://www.nih.gov/od/ord/protocol.htm) scFv: single-chain intracellular antibody.

respectively. We have shown, for instance, that intracellular expression of an anti-erbB-2 single-chain antibody (scFv) results in down-regulation of cell surface erbB-2 expression and selective cytotoxicity in tumour cells expressing the oncogene target both *in vitro* and *in vivo* [8, 9].

TUMOUR PHENOMENA DEPENDENT ON MULTIPLE GENES

Angiogenesis

The development of new blood vessels is a critical step in the growth, progression, and metastatic spread of both solid and haematopoietic tumours. Despite heterogeneity in many other respects, all tumours thus share a universal feature, i.e. they depend absolutely on the vasculature not only to sustain their initial growth and dissemination but also to maintain their long-term viability. Extensive experimental data and clinical-pathological studies support this contention (reviewed in: Special Issue on Angiogenesis, *European Journal of Cancer*, Vol. 32, issue 14, 1996). Vessel targeting, therefore, should be useful for the treatment of most kinds of cancer, either to impede the formation of tumour blood vessels, as initially proposed by Folkman (see Special Issue), or as an attempt to destroy the already formed tumour vasculature, as proposed later by Denekamp and others [10–12]. From the points of view of oncology and gene therapy, two features of this strategy seem most attractive. First, the genetic stability of endothelial cells should essentially eliminate the appearance of resistance to molecular therapeutic interventions [13], which is so pervasive in the treatment of tumour cells. This hypothesis has been confirmed in a cancer animal model that evaluated treatment with the natural angiogenesis inhibitor endostatin [14]. Second, an additional advantage of targeted killing of endothelial cells is the highly amplified killing effect of large numbers of tumour cells when deprived of their vascularisation. This can partially overcome current limitations in the number of cells modified by gene transfer *in vivo*.

In the last decade, anti-angiogenic drugs targeted to the proliferating endothelium of tumours and other diseases have been applied in the clinical setting and have entered clinical trials. In addition, the association of chemo- or radiotherapy with anti-angiogenic agents has been shown to produce an enhanced anti-tumour effect in preclinical models. Notably, combined treatments can achieve cures that are not observed with either treatment alone [15]. Thus, molecular therapeutic interventions against the tumour and its vasculature are not only strongly appealing on theoretical grounds for their use in a variety of clinical contexts, but their utility is also rapidly being tested clinically [16]. Based on this, genetic modification of the endothelium of tumour vasculature has been proposed as an alternative therapeutic modality [17, 18]. With this genetic strategy, the problems of previously explored approaches can be potentially overcome. For instance, local production of high levels of therapeutic proteins can be induced, thus obviating or diminishing the difficulties associated with systemic toxicity, and also pharmacological issues, such as largescale manufacture, bioavailability, and cost of ordinary drugs. In addition, the ability to release the gene product continuously may be relevant in certain cases, such as for the appropriate anti-angiogenic effect of interferon gamma.

Both suppression of angiogenic cellular signals and augmentation of natural inhibitors of angiogenesis have proved to be feasible strategies in *in vivo* tumour models. Examples of effective genetic interventions for the suppression of angiogenesis factors are the down-regulation of vascular endothelial growth factor (VEGF) by antisense molecules, as shown in models of glioma [19, 20], and the blockade of VEGF receptor function by delivery of mutant versions of one of its cognate membrane receptors, Flk-1 [21, 22], or of a secreted soluble version of its other receptor, Flt-1 [23, 24]. In addition, similar results have been obtained by adenoviral-mediated delivery of a soluble receptor analogous to the endothelium-specific Tie2 receptor [25], also known to play a role in tumour angiogenesis. Conversely, the replacement or supplementation of inhibitors of angiogenesis has been attempted by transfecting cells with the thrombospondin gene [26] and by using *in vivo* viral vectors that encode soluble platelet factor 4 [27] and angiostatin [28, 29]. However,

none of these strategies has been clinically tested and major issues, mostly vectorological, are still to be solved. Most obvious are the problems of assuring highly efficient gene delivery and long-term expression of the therapeutic anti-angiogenic genes to keep the tumour deprived of its growth-enabling vascularisation. In addition, the current lack of targetable, injectable vectors impedes the application of anti-angiogenesis gene-based strategies to multiple foci of tumour that characterise disseminated cancer. Lastly, different combinations of endothelial growth factors and their receptors are altered in different tumours, and may even change in single tumours during different stages of progression. Thus, despite its powerful rationale, the successful clinical implementation of anti-angiogenesis gene therapy will still require major developments.

Anti-angiogenesis seems a therapeutic manoeuvre mostly appropriate for avoiding tumour progression but, as mentioned above, alternative anti-vascular strategies have been proposed with the intention of destroying existing vasculature, thereby depriving the tumour of essential vascularisation. To date, there have been few attempts to induce direct toxicity in the vasculature of normal or tumour vasculature by gene transfer [30, 31], but development of targeted vectors should prompt immediate evaluation of such strategies.

Invasion and metastasis

Increasingly, genes and proteins involved in phenotypic aspects of tumours, other than disordered proliferation, are being described and identified as potentially useful therapeutic targets. In this regard, besides angiogenesis, one fundamental component of the metastatic cascade is the local invasion of the extracellular matrix by tumour cells. Studies in animal models have begun to show that modulation by gene transfer of molecules involved in degradation of extracellular matrix, cellular motility, and cellular adhesion, such as plasminogen activators, metalloproteinases and CD44, has the potential for inhibiting tumour cell spread [32]. To have clinical utility, however, these manoeuvres should provide long-term abrogation of the involved molecules and be used when the tumour is going through the earlier steps of the metastatic cascade in a particular patient.

Apoptosis

The highly orchestrated form of cell death known as apoptosis goes awry to some extent in most cancers. Increasingly, a general theme in cancer pathophysiology is the development of a defect in the function of pro-apoptotic molecules, such as p53, that commonly prepare the cell for apoptosis whenever cell proliferation or DNA damage is induced, their absence thus depriving the cell of a critical safety mechanism [33]. Alternatively, a functional excess of anti-apoptotic molecules, such as Bcl-2, may also occur in tumours. In each case, the result is an imbalance that favours the inappropriate survival of tumour cells. The mechanisms involved are attractive therapeutic targets because the tumour cell is totally dependent on them for its survival, and appears to have a higher sensitivity to the induction of apoptosis than normal tissues [33]. In addition, restoring or enhancing the capacity to undergo apoptosis may, in some cases, be a crucial event which renders tumours sensitive to classical anticancer agents, such as chemotherapy [34, 35] and radiotherapy [36, 37].

With the increasing recognition of the molecular basis of the apoptotic pathway [33, 38–40], and the description of several of its components acting as oncogenes or tumour suppressor genes, gene therapy has emerged as a rational strategy for the modulation of apoptosis. Therefore, the genetic modification of tumour cells and their supportive stroma with genes that modulate the apoptotic process has been recently proposed for the gene therapy of cancer [6, 41–43]. Three general requirements for the successful therapeutic application of genetic modulation of apoptosis in cancers are apparent. First, significantly better gene transfer vectors may be needed to modify and trigger apoptosis in most malignant cells in any given tumour. Current vectors are far from achieving *in vivo* the requisite high levels of tumour cell modification. Alternatively, mechanisms may be implemented regionally to amplify the effects of the expression of transferred genes, i.e. by inducing a bystander apoptosis. Second, given the ubiquity of the numerous cellular proteins involved in apoptotic pathways, selective activation in cancer cells of the lethal processes may also be a critical requirement of therapeutic manoeuvres. The lower threshold for undergoing apoptosis that characterises tumour cells could, however, offer an advantageous therapeutic window that makes this requirement less stringent. Third, given the complexity and redundancy of the signalling circuits involved, modulation of several components of the apoptotic pathways may be needed to provoke cell death. Interventions downstream in the circuits might also be preferable to avoid regulatory counterbalances.

Despite the theoretical constraints just mentioned, preliminary attempts to explore the therapeutic modulation of apoptosis against cancer by gene transfer have already begun, driven by encouraging preclinical data in animal models. Clinical trials are currently ongoing evaluating the value of pro-apoptotic *p53* and adenoviral E1A, and a growing number of other candidate genes are being considered and tested preclinically (Table 4).

Obstacles to mutation compensation

Although the strategies currently used for the restoration of normal genes and ablation of mutant genes have offered in-depth insights into the molecular biology of carcinogenesis and tumour progression, they face critical problems that restrict their clinical application. Human tumours are remarkably heterogeneous in the patterns of expression of relevant oncogenes. Thus, therapeutic targeting of a single molecular abnormality may have only an inconsequential impact on the clinical management of the disease, considering both the population and individual patients. In addition, several mutated genes produce molecules with transdominant effects, thus necessitating the blocking of their effects and not merely their supplementation with a wild-type version of the gene. Furthermore, because these strategies mostly modulate intracellular responses, nearly every tumour cell might have to be targeted for these approaches to be clinically effective. The current state of development of gene therapy vectors, both viral and non-viral, makes this feat unachievable within non-toxic margins of vector dose. Clearly, breakthrough developments in vector technology are needed for these obstacles to be overcome. A better understanding of the tumour-supportive micro-environment and of multicellular tumour phenomena may also suggest genetic interventions that, even with a limited gene transfer, can elicit widespread effects in the tumour. In addition, approaches such as molecular chemotherapy or immune system augmentation that exhibit an amplified regional or systemic effect hold the promise of tackling some of the aforementioned limitations.

Table 4. Genetic modulation of apoptosis for cancer therapy

Strategy	Target genes or molecules
Add or restore pro-apoptotic molecules	
Induce exogenous death signals (ligand/death receptor)	Granzyme B/Perforin, Fas1, (CD95L/Fas (CD95), TNF/TNFR1, Apo3L/DR3, Apo2L/TRAIL1/DR4 or DR5
Induce endogenous triggers of apoptosis	Cytochrome c, TP53, *BIA*
Induce pro-apoptosis regulators	bax, bcl-xs, ar-pun
Re-link pro-apoptotic signals with apoptosis effectors	Apoptosome components (cytochrome c, Apaf-1, CARD, caspase-9), others
Activate directly apoptosis effectors	Caspase recruitment domain (CARD)
Suppress anti-apoptotic molecules	
Inhibit exogenous survival signals	/
Inhibit/inhibitors of exogenous death signals *	NF-kB
Inhibit anti-apoptotic regulators	*bcl-2*
	Inhibitors of apoptosis (IAPs), such as survivin, XIAP, IAP-1, and IAP-2

Genes used or suggested in clinical trials appear in italics.

MOLECULAR CHEMOTHERAPY

A number of distinct approaches to molecular chemotherapy for cancer have been developed. These include the administration of (1) toxin genes to eliminate tumour cells and the stromal cells that support them, (2) drug resistance genes to protect the bone marrow from myelosuppression induced by chemotherapy, and (3) genes that enhance the effect of conventional anticancer treatments. Initially, the approach of molecular chemotherapy was designed to achieve selective eradication of carcinoma cells via expression of a toxin gene. This is similar to conventional chemotherapy, where pharmacological agents are employed. However, in the latter approach, toxicity of the drug is often manifested both in malignant and non-malignant cells. Therefore, in order to effect a reduction in the burden of neoplastic cells, the patient's normal tissues and organs have to be exposed to potentially harmful quantities of the drug. Molecular chemotherapy is designed to circumvent this limitation by selectively targeting toxin delivery or expression to cancer cells on the basis of more specific tissue- or transformation-associated markers, thereby reducing the potential for non-specific toxicity. Commonly, a non-toxic pro-drug is administered that requires activation in genetically modified cells in order to be transformed into a toxic metabolite that ultimately leads to cell death [44–49].

Toxin genes

Thymidine kinase. The most common molecular chemotherapy system utilised to date to accomplish cell killing has been the herpes simplex virus thymidine kinase (HSV-*tk*) gene given in combination with the pro-drug ganciclovir (GCV) [50]. The selectivity of the HSV-*tk* system is based on the fact that, contrary to normal mammalian thymidine kinase, HSV-*tk* preferentially monophosphorylates GCV, rendering it toxic to the cell. GCV is then further phosphorylated by cellular kinases to produce triphosphates that are incorporated into cellular DNA. The incorporation of the triphosphate form of GCV causes inhibition of DNA synthesis and of RNA polymerase, leading to cell death [44]. Thus, tumour cells (or any other cell undergoing mitosis) transduced to express the viral *tk* gene have enhanced sensitivity to cell killing after exposure to GCV. Somewhat unexpectedly, normal cells transduced with HSV-*tk* after intravenous (i.v.) [51] or intrahepatic [52] administration of adenoviral HSV-*tk* vector have also shown high sensitivity to GCV, leading to liver degeneration and low survival in mice. The absence of toxicity of GCV after i.v. administration of a control adenovirus or subcutaneous administration of an adenovirus encoding HSV-*tk* suggests that the toxicity is specifically liver-associated. The relationship between toxicity and the status of liver parenchymal cells with respect to the cell cycle remains to be determined. The toxicity and efficacy of the transfer of HSV-*tk* are currently being tested in more than two dozen phase I human clinical trials, including tumours of the ovary, brain, prostate, head and neck, mesothelioma, multiple myeloma, leukaemia, and liver metastasis of colon cancer (for an updated list of protocols visit the Office of Recombinant DNA activities website at http://www.nih.gov/od/orda/protocol.htm).

Bystander effect. Whilst the benefits of selectively eradicating tumour cells are obvious, an important limitation associated with molecular chemotherapy is the inability to genetically modify 100% of the tumour cells with the toxin gene. However, this has proved not to be as severe a limitation as initially thought due to the phenomenon known as the 'bystander effect', whereby the eradication of HSV-*tk* transduced cells elicits a killing effect upon the surrounding non-transduced tumour cells. That not all of the tumour cells need to contain the HSV-*tk* gene to obtain complete eradication of the tumour was an observation of early experiments employing the relatively inefficient retroviral vectors in brain tumours [53, 54]. This occurrence was later confirmed in a variety of other tumour model systems [55–58].

Other toxins. Several additional combinations of enzyme/pro-drug have been developed to improve the efficacy of molecular chemotherapy and overcome the limitations of *tk*/GCV. For example, some of the enzyme/pro-drug combinations induce toxic effects not only in cycling but also in non-cycling cells (carboxypeptidase G2, nitroreductase, purine nucleoside phosphorylase). With others, the bystander effect is stronger (purine nucleoside phosphorylase) or does not require cell contact (cytosine deaminase, nitroreductase).

With some exceptions, single drugs in standard chemotherapy do not cure cancer. Historically, effective cancer treatments were developed when drugs with different mechanisms of action were used in combination. Extending

this concept to molecular chemotherapy, several combinations of enzyme/pro-drug have been shown to induce synergistic killing effects *in vitro* [59,60]. Combination schemes have achieved also higher rates of tumour regression and cure in animal models [61,62]. Thus, the application of classical chemotherapy principles for designing drug combinations would recommend the use of pro-drug/enzymes that target both dividing and non-dividing cells, that elicit different mechanisms of bystander effect, and that have non-overlapping toxicities.

Drug-resistance genes

In a second molecular chemotherapy approach, the host tolerance to higher doses of standard chemotherapeutic drugs is increased by transducing bone marrow cells, known to be highly sensitive to chemotoxicity, with genes that confer drug resistance [63–65]. Some potential problems with this strategy are, however, apparent. These include the absence of clear cut evidence demonstrating that higher chemotherapy doses translate into improved patient survival, very low transduction efficiency of the target human haematopoietic cells with retrovirus vectors, the dose-limiting effects determined by other non-haematological toxicities, and the fact that contaminating cancer cells in the marrow could be transduced with the drug-resistance gene, which could rapidly give rise to clones of treatment-resistant tumour cells.

Chemosensitisation and radiosensitisation

A third approach of molecular chemotherapy seeks to modulate the level of expression of a variety of genes that influence the sensitivity of the cell to toxic stimuli, including conventional chemotherapeutic drugs and radiotherapy. Genetic chemosensitisation can be achieved by inducing apoptosis, by inhibiting molecules involved in tumour cell resistance, or by enhancing intratumoral production of cytotoxic drugs. To facilitate apoptosis, genes such as *p53* may be administered to tumour cells to enhance the mechanisms of apoptosis induced by chemotherapeutic agents [66]. Our group has shown that down-regulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increases drug-induced cytotoxicity [67]. Analogously, genetic down-regulation of cellular factors related to chemoresistance has been shown to enhance chemosensitivity [68]. Alternatively, genes can be administered intratumorally to enhance metabolic conversion of conventional chemotherapeutic agents. For example, transfer of a liver cytochrome P450 gene, *CYP2B1*, into human breast cancer cells greatly sensitised these cells to the cancer chemotherapeutic agent cyclophosphamide as a consequence of the acquired capacity for intratumoral drug activation. This effect produced a substantially enhanced antitumour activity *in vivo* [69]. Lastly, combinations of conventional chemotherapeutic agents and molecular chemotherapy can serve the established rule of administering cytotoxic drugs with different mechanisms of action and toxicities. For example, one ongoing clinical trial is evaluating the association of adenovirus-mediated transduction of ovarian cancer cells with the *tk* gene followed by administration of acyclovir and the chemotherapeutic drug topotecan (http://www.nih.gov/od/orda/protocol.pdf).

Several drugs are proven radiosensitisers, a fact that is commonly exploited in the clinic. One of these drugs is 5-fluorouracil (5-FU), which can be produced by the cytosine deaminase (CD) suicide gene. In this regard, molecular chemotherapy based on CD has been shown to enhance the effects of radiation therapy in animal models of gliosarcoma and cholangiocarcinoma [70]. Thus, strategies to alter both chemosensitivity and radiosensitivity by gene transfer appear to have potentially wide applicability in many tumour contexts.

Obstacles to molecular chemotherapy. With all its promise, molecular chemotherapy also bears some practical limitations. To date, the strategy of molecular chemotherapy has been mainly used in loco-regional disease models to overcome the lack of targeted vector systems. In these *in situ* schemes, a vector encoding the toxin gene is administered intratumorally or into an anatomic compartment containing the tumour mass. The goals of this delivery method are to achieve high local vector concentration in order to favour tumour transduction and to limit vector dissemination. However, transduction efficiencies of presently available vectors have been shown to be inadequate. Even in the context of closed compartment delivery, it has not been possible to modify a sufficient number of tumour cells to achieve a clinically relevant tumoral response [71]. Furthermore, although transduction with HSV-*tk* followed by ganciclovir treatment reduces tumour burden and prolongs survival in various model systems, including those utilising intratumoral and intraperitoneal (i.p.) administration, the elevated doses of viral vector needed to obtain transduction of the majority of the tumour cells are associated with limiting toxicity. In fact, substantial toxicity and experimental animal death have been noted [51,52,72]. Thus, the small therapeutic index of currently available vectors in the context of *in situ* administration is a critical limiting factor for the purpose of gene therapy of cancer. Furthermore, and most importantly, a well-known limitation of conventional chemotherapy is also to be expected with the use of molecular chemotherapy, i.e. the appearance of drug-resistant tumour subpopulations (Table 1). In conclusion, vector limitations and well-known barriers to classical cytotoxic manoeuvres impede the full exploitation of the promise of a more selective eradication of carcinoma cells via the expression of toxin or protective genes.

GENETIC IMMUNOPOTENTIATION

The development of clinically evident tumours implies the obvious failure of the host immune system to recognise and eliminate tumour antigen(s), a hypothetical role suggested by Thomas and embodied by Burnet under the name of 'immune surveillance of neoplasia' [73]. Genetic immunopotentiation strategies attempt to achieve active immunisation against tumour-associated antigens by gene transfer methodologies applied either to tumour cells, to enhance their immunogenicity, or to cellular components of the immune system, to enhance their anti-tumour prowess.

Genetic modification of immune effector cells

Cells of the immune system have been modified to augment their capacity to recognise and reject tumour antigens [74]. To this end, gene therapy offers the possibility of genetically modifying effector cells and, importantly, this intervention can be performed *ex vivo*, thus avoiding the toxicity that characterises most biological response modifiers when administered systemically.

Tumour infiltrating lymphocytes (TILs). TILs are derived from mononuclear cells obtained from leucocytes infiltrating resected specimens of solid tumours. In the early 1990s, it was hypothesised that TILs could be an enriched source of

natural killer (NK) cells and cytotoxic T-lyphocytes (CTLs) specific for tumour antigens, and could also have tropism towards systemic tumour foci. On this basis, technology for their expansion in culture was developed, and TILs were the first immune cells to be genetically modified and applied in a human gene therapy clinical trial against cancer [75]. It was soon observed that while TILs do include CTL and NK activated cells, only a few of these cells in these mixed populations are specific against the tumour from which they are isolated. Furthermore, reinfused TILs localised poorly into tumours, and their required expansion *in vivo* using IL-2 was rather toxic. Although several strategies have been applied to improve treatments based on TILs and other lymphocytes, including an elegant re-engineering of their tropism [76, 77], a modest localisation of TILs in tumours remains a limitation for the efficacy of this poorly tolerated and expensive therapy.

Genetic modification of tumour cells

An alternative strategy for trying to augment the anti-tumour immune response is to genetically modify tumour cells, or to manipulate their components, to facilitate the start of a robust immune response. Thus, it has been hypothesised that a formerly tolerant host may revert its immune status, characterised by tolerance or anergy, and thus ultimately experience tumour rejection. In other words, it is hypothesised that the host can be 'vaccinated' against the tumour by exposing tumour antigens to the immune system in a more favourable context [78–81]. Most clinical experience with antitumour vaccines to date has been obtained in melanoma patients. For years, irradiated tumour cells, either autologous or allogeneic, were administered in combination with different adjuvants, such as BCG. Later, the molecular definition of tumour-associated antigens allowed the testing of vaccines based on individual antigenic determinants delivered to the patient in the form of peptides or DNA. More recently, tumour cells themselves have been genetically modified to increase their immunogenicity by transfer of a variety of genes, including cytokines such as GM-CSF, costimulatory molecules such as B7, and MHC molecules. Clinical responses have been occasionally observed in melanoma, but not in colon or renal cancer [82]. A common requirement, not adequately accomplished routinely yet, is to introduce the gene of interest in tumour explants or cultured cells with high efficiency. A more fundamental problem has been observed in experimental models using tumours naturally arising in transgenic mice. In these spontaneous tumours, a clear lack of efficacy of vaccines called into question the relevance of previously observed responses in animal models of grafted, syngeneic tumours [83].

Obstacles to genetic immunopotentiation

The main advantage of genetic immunopotentiation is the possibility of enlisting physiological mechanisms for a potentially vast amplification of the therapeutic manoeuvre. To this end, even modest levels of gene transfer were initially expected to be followed by clonal expansion and systemic spread of effector immune cells and mediators. Thus, efficiency of gene transfer would be not critical, given the relatively low amounts of cells and gene products needed to obtain a potentially powerful response from the immune system.

The level of gene transfer into tumour and immune effector cells observed clinically has been limited [82], and this has been thought to partly explain the poor results obtained by tumour immunotherapy in humans. However, there are other, probably more important, obstacles. Factors that can explain the failure of the immune system in the cancer patient are legion, and it is not clear which of them are critical in the clinical context. Some of these factors may similarly explain the failure of previous immune therapeutic attempts. In general, a lack of an immune response can be due to inadequate immunogenicity of the tumour or to a deficiency of the immune system to recognise, respond and reject tumour antigens. Reduced tumour immunogenicity can be related to the absence, heterogeneity and plasticity of tumour-specific antigens or the loss of MHC class I molecules on the tumour cells, which are essential for presentation of cellular antigens to effector $CD8^+$ T lymphocytes. Alternatively, it may well be that the lack of costimulatory molecules, such as B7, in tumour cells and the lack of other 'danger' signals in the tumour site establishes immune tolerance or ignorance, which keeps the tumour from being rejected. In effect, current knowledge of tumour immunobiology establishes that T cells able to recognise tumour-associated antigens can be found *in vivo* and are inducible *in vitro*. Thus, the lymphocyte repertoire against these epitopes has not been deleted. However, either tolerance to these (tumour) self-antigens has been induced or, in the absence of costimulatory signals, peripheral T cells simply have ignored these antigens or become tolerant ([84] and discussion below on the 'danger' model). In this regard, induction of tumour antigen-specific T cell anergy in adoptively transferred cells has recently been shown in experimental models to be an early event in the course of tumour progression [85]. In addition, studies with transgenic mice that develop spontaneous tumours have shown that vaccination with tumour cells transduced with cytokines fails to inhibit tumour onset and progression, whereas the same cells are able to immunise non-transgenic mice subsequently grafted with tumours [83]. Thus, the failure of naturally established tumours to present antigens efficiently, and to attract and activate tumour-specific T cells at the tumour site, may impede successful vaccination against cancer antigens. Of note, ignorance by the immune system can abort most of the immunotherapy manoeuvres being tested and discussed above. An obvious consequence is that cancer vaccines should be able by design to break down tolerance to tumour antigens.

Immune system deficiencies can, in turn, be either generalised or regional, including in the latter case the active suppression by the tumour of host antigen presentation and of effector cells in the local micro-environment by expression of a variety of molecules. (For reviews on the mechanisms involved in tumour escape see refs. [86, 87].) Clearly, the presence of immunosuppressive factors in tumours suggests the need to complement any immunotherapy strategy with manoeuvres explicitly addressing the intratumoral presence of inhibitors of the immune system response, a combined strategy which to our knowledge is yet to be directly tested. An additional general feature of the immune response to consider when designing gene-based immunotherapy is the redundant phenomenology of the immune system. Its destructive power, occasionally needed in its entire exuberance, requires a complex network of balances and counterbalances to control the pathways of activation and termination of the immune response. Interventions directed to supplement or inhibit single mediators will most probably yield partial physiological and therapeutic results in the best case, may frequently yield no result at all, and occasionally

will produce effects opposed to those desired. Thus, combinations of cytokines are increasingly being used to try to control the complexity of the immune response against tumours. In the field of organ transplantation, successful induction of tolerance to prolong organ survival has been achieved by blocking multiple effector cells and mediators of the adaptive and innate immune systems. Similarly, it is conceivable that breaking the tolerance to tumours will require a strategy of multiple interventions including several target cells and cytokines.

NOVEL STRATEGIES TO OVERCOME CURRENT LIMITATIONS

As we have reviewed above, gene transfer therapies are remarkably successful in *in vitro* and *in vivo* animal model systems. In effect, we already know that the malignant phenotype can be reverted in tumour cell lines by 'knocking-out' or adding certain genes; that tumours can be eradicated by delivery of cytotoxic genes followed by treatment with appropriate pro-drugs; and that tumours can be cured in murine models by making the tumour cells either more immunogenic or by making the immune system cells more responsive, via the expression of cytokines, or by induction of costimulatory and immunogenic molecules. However, overriding limitations have been made apparent in pre-clinical experiments and in the first human gene therapy clinical trials against cancer, as emphasised by the Orkin-Motulsky report to the NIH [88] and the first published clinical results. Most difficulties in obtaining clinically relevant benefits come from the inefficiency of current gene vectors in transducing tumour or immune cells and their inability to access in a selective way target cells distributed systemically. Several avenues for improvement have been proposed, and some will be succinctly reviewed in this section.

Mutation compensation requires quantitative gene transfer

For mutation compensation strategies to work successfully, it seems that every tumour cell would have to be corrected in its genetic defect to achieve a therapeutic outcome. Thus, quantitative transduction of therapeutic genes into the tumour after *in situ* administration of the gene therapy vector may be an essential requirement. To this end, a variety of vector amplification strategies are being explored, including replicative [89, 90] and integrative [91] viral systems.

Replicative vector systems. One method to circumvent suboptimal tumour transduction of therapeutic genes *in vivo* would be the use of conditionally replicative viral vectors: a replication-competent virus would be employed to replicate selectively within infected tumour cells, leaving normal tissues unaffected. Production of progeny virions from the infected tumour cells would then allow infection of neighbouring tumour cells. Thus, the intratumoral viral inoculum would increase, improving the tumour transduction efficiency. In addition, the use of viruses that display a lytic life cycle would allow virus-mediated oncolysis. This effect would occur irrespective of the delivered transgene. In both cases, an amplification of the antitumour effect would be achieved [90, 92]. The limitations of non-replicative vectors already observed in human trials have facilitated rapidly increasing acceptance of this experimental strategy, once considered an eccentric endeavour.

For clinical application of this strategy, a virus with *in vivo* stability and the capacity for conditional replication within tumour cells is mandated [93]. Lack of integration of the viral genome into the cell chromosome seems also desirable. In this regard, both recombinant adenoviruses and herpes viruses have the potential to provide the required properties. Not only do they display high efficiency and stability *in vivo*, but also their replication can be controlled. In the case of adenoviruses, replication can be restricted to tumour cells by placement of genes needed for viral replication under the control of tumour- or tissue-specific transcriptional control elements, such as the promoter of the prostate-specific antigen (PSA) [94]. Alternatively, mutant adenoviruses have been designed to replicate selectively in cells lacking functional *p53*. Because *p53* is absent in many tumours, the replication of this lytic adenovirus would be selective in tumours, and a therapeutic strategy for cancer based on this concept has been proposed [95]. Clinical trials using this virus are currently ongoing, and encouraging preliminary results have been presented [96]. However, extensive studies in a variety of cell lines and animal tumour models have to date failed to confirm the selective properties of the virus to replicate only in *p53* mutant tumour cells [97, 98].

Herpes viruses have also been developed that replicate conditionally in dividing or tumour cells. This selectivity is based on several possible mutations engineered in the viral genome that prevent it from replicating unless the infected cell provides for a substituting molecular activity [99]. These properties have established brain tumours, which are surrounded by non-mitotic cells, as an ideal therapeutic model for testing replication-conditional herpes vectors. Notably, clinical trials have already begun to test both adenovirus and herpes virus-based replicative vector systems for the treatment of human cancer.

A small, non-pathogenic virus called parvovirus went through human trials of viral oncolysis several years ago. The ability of this virus to replicate depends on factors associated with proliferation and differentiation, and as a consequence the virus preferentially displays a cytopathic effect in transformed cells. However, the capacity of the virus to replicate and spread robustly within a solid tumour, and subsequently to induce tumour lysis, appears to be limited.

As another intriguing example, the capacity of human reovirus to replicate selectively in tumour cells having an activated Ras signalling pathway has recently been described in an *in vivo* model [100].

Further refinements in replicative vectors are anticipated that can significantly enhance the possibilities for the realisation of a practical clinical benefit in the context of virus-mediated cancer treatment. A systematic analysis of the life cycle of a replicative virus reveals four areas where further engineering of vectors can bring the required improvements. Thus, better vectors would have increased infectious capacity, would replicate with tight selectivity in target tumours or tissues, would have an enhanced replicative 'burst', and would modulate the local immune response allowing unimpeded regional dissemination throughout the tumour to the required extent. Efforts to realise each of these features have already begun in several laboratories [101–103]. As early examples, our group is developing defective adenoviral vectors that replicate selectively under the stimulus of the cytokine interleukin-6 [104], or under the controlled addition of second vectors carrying replication-enabling DNA sequences [105, 106].

Prolonged transgene expression: integrative vector systems. Lack of stability *in vivo* has confined the use of retroviruses to the *ex vivo* modification of target cells. For *in situ* gene delivery, vectors with high efficiency and stability *in vivo* are needed. Of vector systems with both characteristics, adenoviruses have been most extensively characterised and used (Table 5). However, adenoviruses also have important limitations. In addition to a significant inflammatory and immune response, an additional basis for the limited transgene expression associated with adenoviral vectors derives from their non-integrative nature, such that vector sequences are not retained in the host genome and are not inherited by progeny cells. In this regard, after adenoviral-mediated gene transfer, the recombinant genome is present as an episome in infected cells. Thus, with the proliferation of transduced cells, vector sequences are lost, with the consequence of limited duration of transgene expression. For utility in mutation compensation, and in other gene therapy strategies it thus would be desirable to develop methods to achieve integration of adenoviral vector-delivered transgene sequences in infected cells. As a novel approach to meet this need, we and others have developed a chimeric viral vector system that exploits favourable aspects of both adenoviral and retroviral vectors. In this scheme, adenoviral vectors induce target cells to function as transient retroviral producer cells *in vivo*. The progeny retroviral vector particles can then effectively achieve stable transduction of neighbouring cells [107, 108]. Thus, the principle of combining selected features of available vectors into novel chimeric vectors is being explored in the development of virus-based gene transfer systems [109].

Lentiviruses are retroviruses that, in contrast to other members of the family, can infect both dividing and non-dividing cells. This fundamental feature has driven significant efforts for the development of recombinant lentiviral vectors, although practical issues related to the production and safety have to date limited its widespread use. The recent development of novel vector packaging systems can significantly facilitate availability [110], and new self-inactivating lentiviral vectors can allow safer use [111, 112]. Efficiency of transduction of potential cellular targets by pseudotyped lentiviral vectors and *in vivo* utility are intriguing, and have begun to be described [112, 113].

Prolonged transgene expression: immune tolerance to viral vectors. Gene delivery via adenoviral vectors has been associated *in vivo* with the induction of characteristically intense inflammatory and immunological responses. A number of specific cellular and humoral immune effector mechanisms, together with non-specific innate defence factors, eliminate the infecting virus [114–117]. This process, refined over the course of millennia for maximal efficiency, has been associated with attenuation of expression of the transferred therapeutic gene due, at least in part, to loss of the vector-transduced cells. Based on an understanding of the biology of this phenomenon, specific strategies have been developed to mitigate the process [103]. Of note, the recent development of replicative viral vector systems will mandate the effective modulation of the anti-viral immune response.

Manoeuvres to minimise the immune response against viral vectors include manipulations of both the vector and the host. Firstly, recombinant viral vectors can be genetically engineered to delete viral genes encoding highly immunogenic or cytotoxic viral proteins. However, viral vectors with most of their genomes deleted are more difficult to propagate and purify, transgene expression tends to be unstable, and the vectors are still not totally devoid of immunogenic properties. However, the most recent versions of these vectors may provide adequate production and non-toxic, sustained expression of encoded genes for several months [118]. Alternatively,

Table 5. Gene transfer systems used clinically against cancer

Type	Vector system	Duration of expression	Clinical trials (No.)	Distinguishing features
Nonviral	Liposomes	Transient	30	Repetitive and safe administration feasible, inefficient gene delivery, transient expression.
	Naked DNA or RNA (injection, gene gun, electroporation)	Transient	5	Easy preparation, inefficient gene delivery, transient expression.
	Molecular conjugates	Transient	–	Flexible design, inefficient gene delivery, transient expression, unstable *in vivo*.
	Bacteria	–	–	Useful as vaccines for gene delivery into antigen presenting cells.
Viral	Retrovirus	Prolonged	63	Integrates into the chromosome of dividing cells, unstable *in vivo*.
	Adenovirus	Transient	34	Highly efficient *in vivo*, production in high titre, tropism can be modified, induces potent inflammation and immunity, replicative vectors available.
	Poxvirus (vaccinia)	Transient	15	Extensive clinical experience with parent virus, large insert capacity, induces potent inflammation and immunity.
	Adeno-associated virus	Prolonged	–	Non pathogenic, low insert capacity, difficult to scale-up.
	Herpes simplex virus	Transient	1	Highly efficient *in vivo*, large insert capacity, cytotoxicity replicative vectors available.
	Chimeric vectors (e.g. adr/retro)	Prolonged	–	Combine features of component genetic vectors.
	Lentivirus	Prolonged	–	Integrates into the chromosome of *both* dividing and non-dividing cells, well-characterised production system not yet established.

*Registered in the NIH Office of Recombinant DNA Activities in December of 1998 (http://www.nih.gov/od/orda/protocol.pdf)

different serotypes and species of adenoviruses have been proposed to minimise the stimulus for an immune response. Secondly, vectors have been modified to express immunomodulatory molecules. It has been hypothesised that this could create a locally privileged environment for the vector. Some of these engineered molecules are viral genes that interfere with the apparatus of antigen presentation [119], such as the adenoviral glycoprotein 19 K, the herpes simplex virus (HSV) immediate early protein ICP47, or the viral interleukin 10 [120]. Others are recombinant molecules designed to imitate the viral proteins mentioned, such as antisense oligonucleotides or single-chain antibodies against MHC class I and II proteins, or to block costimulation, such as CTL4Ig [103].

Interventions on the immune system of the host have been adopted from common practices in the field of organ transplantation. In this regard, virally transduced cells have been considered to behave, to some extent, as allogeneic cell transplants. Thus, drugs are employed that inhibit the cellular immune response, such as anti-CD4 antibodies, cyclosporine, dexamethasone, and FK 506. In addition, drugs that decrease the humoral immune response, such as cyclophosphamide and deoxyspergualin, have been used. Recently, several groups have demonstrated transient and more specific immune blockade with inhibitors of T cell costimulation, such as anti-CD40 ligand, CTL4Ig, and anti-LFA-1. Furthermore, interventions aimed to decrease the innate response have recently been attempted. For instance, a soluble tumour necrosis factor receptor has been shown to greatly reduce the early adenovirus-induced inflammatory response, and to prolong expression of encoded genes [121]. Unfortunately, the required chronic administration of these immunosuppressive drugs affects systemic immune function and could lead to a number of potential complications, such as infection and malignancy. This makes them less attractive in principle for clinical application, although short-term treatment in cancer patients should be feasible. Lastly, a more specific intervention, induction of tolerance to adenovirus vectors, has been induced by several manoeuvres, including intrathymic injection of adenovirus [122], oral ingestion of adenoviral antigens [123], and infusion of antigen-presenting cells infected with adenovirus and expressing Fas ligand [124]. Thus, although inflammatory and immunological issues have limited the overall utility of adenoviral vectors for gene therapy applications, many of the aforementioned strategies appear promising, and may ultimately allow these limitations to be overcome, at the very least in the context of cancer treatments.

Molecular chemotherapy requires selectivity and amplification

Any approach to cancer gene therapy involving either molecular chemotherapy or mutation compensation requires a high level of efficiency of gene transfer specifically to the tumour cells. Selective gene delivery is necessary because the number of vector particles available for delivery to the cancer cells would be decreased by sequestration by normal, non-target cells. This would then allow ectopic expression of the delivered therapeutic gene, with possibly deleterious consequences for the normal cells [125].

To date, *in vivo* cancer gene therapy strategies have been restricted to the treatment of compartmentalised tumours in an attempt to achieve high local vector concentrations and relatively efficient tumour transduction. Thus, molecular chemotherapy has been employed in a number of animal models and clinical trials in which adenoviral or retroviral vectors or retroviral vector-producing cells expressing a toxin gene have been directly injected into localised neoplasms confined within body cavities [72, 126–129]. The tumours treated in this manner include glioblastoma, mesothelioma and ovarian carcinomas.

However, these attempts to restrict expression of the therapeutic gene to the target cancer cells merely by confining vector administration have proved inadequate. In this regard, locally administered adenoviral vectors carrying the HSV-*tk* gene have been shown to disseminate, probably as a result of leakage into the blood stream, resulting in a high level of liver-associated toxicity [51]. Substantial hepatic toxicity related to the absence of tumour cell-specific targeting has also been demonstrated in adenovirus-mediated transfer of the HSV-*tk* gene in an ascites model of human breast cancer [72]. In addition, *in situ* injection of adenoviral vectors has been associated with a low level of efficiency of gene transfer to the disease cells in human clinical trials [71]. This phenomenon has been correlated with a paucity of primary receptors on the cancer cells [101, 130]. Hence, it is apparent that there is a need to develop a vector which will achieve a high efficiency of gene transfer selectively to target tumour cells following compartmentalised administration in order to increase the therapeutic index and realise the full potential of gene therapy as a safe approach to the treatment of cancer. Moreover, it is clear that the presently available vectors are inadequate for the treatment of metastatic disease. In order to achieve gene delivery to disseminated cancer cells, the vector must be administered i.v. In this context, there is a stringent demand for specificity of gene delivery to the tumour cells, both in order to avoid vector wastage following transduction of nontarget cells and, more importantly, to prevent toxicity associated with expression of the therapeutic genes in normal cells [125]. Therefore, a means must be developed to modify the gene delivery vehicle to permit efficient gene expression specifically in target cancer cells.

Targeting. Targeted gene therapy for cancer can be accomplished at different levels [131]. In one approach, the tumour cell can be targeted at the level of transduction to achieve the selective delivery of the therapeutic gene. This involves the derivation of a vector that binds selectively to the target cancer cell. Alternatively, the therapeutic gene can be placed under the control of tumour-specific transcriptional regulatory sequences that are activated in tumour cells but not in normal cells and, therefore, target expression selectively to the tumour cell. In addition, targeted cancer gene therapy can exploit the unique physiology of solid tumours.

Transductional targeting. The ability to alter the binding tropism of viral vectors is based on an understanding of the basic biology of viral entry. In this regard, attempts to modify the tropism of adenoviral vectors have been facilitated by the fact that the entry of adenoviruses into susceptible cells requires two sequential steps involving the interaction of two distinct viral capsid proteins with specific receptors on the surface of the target cell. The initial high affinity binding of the adenovirus to the primary cellular receptor (designated the coxsackievirus and adenovirus receptor, CAR [132, 133], occurs via the carboxy-terminal knob domain of the fibre [134, 135]. The next step in infection is internalisation of the virion by receptor-mediated endocytosis potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base with secondary host cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [136, 137].

Therefore, strategies to alter adenoviral tropism are based on modification of the viral capsid proteins, fibre and penton base, to permit the recognition of alternative cell-specific receptors. To this end, we have shown that it is possible to redirect adenoviral infection by employing the Fab fragment of a neutralising anti-knob monoclonal antibody (MAb) chemically conjugated to a cell-specific ligand [138–144]. When complexed with preformed adenoviral vector particles, the bispecific conjugate simultaneously ablates endogenous viral tropism and introduces novel tropism, thereby resulting in a truly targeted adenoviral vector. We have employed a number of targeting ligands, including folate, basic fibroblast growth factor, and an antibody directed against the epidermal growth factor receptor. In this manner, we have demonstrated that tropism-modified adenoviral vectors can infect cells that are refractory to transduction by the native vector; that tropism-modified adenoviral vectors can enhance gene transfer to target cells; and that this enhancement in infection can be translated into a therapeutic benefit *in vivo*. Wickham and colleagues have similarly retargeted adenoviral vectors by means of bispecific antibodies, in this case comprising a MAb to an epitope engineered in the penton base and a MAb to a cell surface receptor [145, 146]. However, this approach to the generation of tropism-modified adenoviral vectors suffers from a number of limitations. In particular, since the targeting ligand is not covalently coupled to the adenovirus particle, there is the potential for the bispecific conjugate to become dissociated from the vector.

The drawbacks inherent in any strategy to redirect adenovirus tropism by complexing the vector particles with bispecific targeting conjugates could be avoided by the direct genetic engineering of the viral capsid proteins to contain cell-targeting ligands. In this regard, the carboxy terminus of the adenovirus fibre protein can be modified to incorporate targeting motifs with specificity for cellular receptors [147–150]. In an alternative approach, it has also been reported that targeting ligands can be incorporated within the so-called HI loop of the fibre knob [101, 151]. Adenoviral vectors which have been engineered to incorporate either a polylysine motif at the carboxy terminus of the fibre [147, 150] or an RGD motif at the carboxy terminus [149] or in the HI loop [101] have demonstrated significantly enhanced infection of cancer cell lines and primary tumour cells which express low levels of the primary adenovirus receptor. Thus, these genetic modifications to the fibre protein have resulted in expanded tropism by successfully redirecting adenovirus binding to alternative cellular receptors.

The next challenge in this field will be to employ genetic methods to engineer adenoviral vectors with specificity for a single target cell type. In addition to recognising novel receptors, such vectors should also lack the ability to bind to the native primary adenovirus receptor. This could be accomplished by site-directed mutagenesis of the fibre knob domain to eliminate the cell-binding site. An important consequence of the ablation of native adenovirus tropism is that it will not be possible to propagate these vectors on standard cell lines that express the fibre receptor. However, we have recently developed a novel artificial primary receptor that can be recognised by adenovirus vectors that fail to bind the native fibre receptor [152]. This technology should be useful in the propagation of genetically modified, truly targeted adenoviral vectors.

In contrast to adenoviruses, retroviruses employ a single envelope glycoprotein to accomplish both binding to the cellular receptor and the subsequent step of membrane fusion. As a consequence, modification of retroviral tropism has proven problematic, with few reports of modified envelope proteins which retain these two functions of binding and fusion [153]. A number of molecules, including single-chain antibodies, growth factors and cytokines, can be genetically incorporated into the retroviral envelope glycoprotein, whereupon they confer novel binding specificities into the engineered viral particles. However, some of these surface displayed polypeptides failed to mediate retroviral infection; rather, they proved inhibitory to gene delivery by the modified vectors. In an elegant approach to overcome this obstacle, Russell has incorporated a protease cleavage site into the design of the retargeted vector. Thus, upon contact with proteases expressed on the surface of the target cell, the inhibitory polypeptide is cleaved from the viral surface, thereby restoring infectivity. To date, tropism-modified retroviral vectors have suffered from significantly lower viral titres than the parental vectors and it is therefore not yet proven possible to employ targeted retroviruses *in vivo*.

A key factor in any transductional targeting scheme is the availability of appropriate specific molecules on the target cells that can be exploited. To date, a somewhat restricted range of targeting moieties have been chosen either for proof of principle or for their ability to bind to the relatively short list of previously identified cellular receptors. However, a number of groups have described systems which fundamentally share the similarity of examining libraries of peptides displayed on the surface of bacteriophage for their ability to bind to specific cell types, both *in vitro* and *in vivo* [154, 155]. Thus, a powerful new technology has been developed which allows the rapid isolation and screening of potential tumour-specific ligands, without requiring that the target of the ligand be identified. This approach should, therefore, prove to be a high throughput method to permit the derivation of transductionally targeted vectors for cancer gene therapy.

Transcriptional targeting. Transcriptional targeting has found wide application in the area of molecular chemotherapy where tumour- or tissue-specific regulatory sequences have been employed to restrict expression of the prodrug-converting enzyme specifically to the target cancer cells. For example, transcriptionally targeted adenoviral vectors expressing toxin genes under the control of the tumour-specific alpha-fetoprotein promoter have been employed in molecular chemotherapy approaches to hepatocellular carcinoma [156, 157]. The selective expression of the therapeutic gene in the target hepatomas suggests that transcriptionally targeted adenoviral vectors would be of clinical utility in other diseases. However, it has been reported that certain tumour-specific regulatory elements lose their specificity in the context of an adenoviral vector. Further limitations come from the prohibitively large size of many regulatory sequences, which exceed the capacity of certain current vectors. However, novel gene transfer systems with larger capacity are being developed and could be employed to overcome this limitation—these vectors include gutless adenoviral vectors [158] and reviewed in [117] and recombinant herpes virus [159].

To date, targeted gene therapy has been attempted by employing either transductional targeting or transcriptional targeting alone. However, it should be possible to enhance the overall level of specificity by combining the complementary

approaches of transductional and transcriptional targeting, each of which might be imperfect or 'leaky' by itself [131].

Targeting strategies exploiting tumour physiology. As described above, current approaches to targeted gene therapy for cancer have exploited cellular and molecular differences between normal and malignant cells. However, the physiology of solid tumours at the micro-environmental level provides a unique and selective target for cancer treatment [160, 161]. The regions of hypoxia and necrosis within solid tumours present opportunities for targeted, tumour-selective gene therapy. For example, the hypoxic environment of solid tumours provides a selective means to control gene transcription based on lower oxygen levels compared with normal tissues. Gene therapy strategies activated by hypoxia could include the transcriptional control of a prodrug-activating enzyme by a hypoxia-responsive element. Of course, this approach will still require a means of delivering the constructs specifically to the tumours. Gene therapy strategies could similarly be designed to exploit tumour necrosis. In this regard, certain species of anaerobic bacteria of the genus *Clostridium* can selectively germinate and grow in hypoxic/necrotic regions of solid tumours after i.v. injection of spores [162]. Thus, it might prove possible to exploit clostridia as gene therapy vectors engineered to express therapeutic genes, e.g. a prodrug-activating enzyme.

Modulation of the bystander effect. Limitations of current vectors preclude direct genetic modification of a significant proportion of malignant cells in tumours. It is, therefore, of paramount importance for obtaining clinically relevant results to extend the effects of therapeutic gene expression from the transduced cells to neighbour non-modified cells. Several manoeuvres may be undertaken to extend the magnitude of this required bystander effect. First, survival of genetically modified cells can be prolonged. By doing this, modified cells can sustain longer the expression of the therapeutic gene, thus enhancing the exposure of bystander cells to its protein product. For example, the expression of the cyclin-dependent kinase inhibitor p27 inhibits DNA synthesis and, thus, renders the cells resistant to concomitant herpes simplex virus thymidine kinase/ganciclovir (HSV-*tk*/GCV) treatment. These cells with augmented survival are thus, allowed to prolong the time during which they can pump out cytotoxic metabolites, and hence the bystander effect is increased [163]. However, this intervention should not compromise the capacity for eradication of the genetically modified cells, which could dangerously equate this strategy to the genetic induction of resistance to treatment. Second, the definition of the molecular basis of the bystander effect allows novel interventions to increase directly its magnitude. The intercellular gap junctions, for instance, are known to mediate at least in part the bystander effect of HSV-*tk*/GCV treatment. Retinoic acid and the drugs apigenin and lovastatin upregulate the function of the gap junctions, and have recently been shown to increase considerably the killing effect of HSV-*tk*/GCV both *in vitro* and *in vivo* [164, 165]. Conceivably, genes that encode gap junction molecules can also be transferred into tumour cells for increasing the bystander effect. Third, it is possible to employ therapeutic genes that can be secreted and exert their function in an autocrine and paracrine manner, thus extending regionally their effects against the tumour or its supporting stroma. For instance, the secretion of a soluble receptor for an essential angiogenesis factor can compete regionally for the natural receptor. This blockade limits binding of the angiogenic growth factor to the natural receptor, and consequently restricts the development of the tumour vasculature, thus leading to tumour suppression [23, 24]. There is, therefore, accumulating evidence that the modulation of the bystander effect can regionally amplify the effects of therapeutic gene transfer, and can contribute to overcoming the limitations of current vector systems.

Cellular vehicles. Vectors with the capacity for targeted systemic gene delivery have not been available, and this fact has limited the overall efficacy of gene therapy in cancer, including molecular chemotherapy strategies. As an alternative to viral and other nonviral vectors, cells have been employed for gene delivery. In this approach, the cells are removed from the body and therapeutic genes are transferred into them extracorporally, followed by autologous re-implantation into the patient. In this manner, the genetically modified cell becomes itself the ultimate vector for gene delivery. Examples of primary cells commonly used in this context, so-called 'cellular vehicles', are T lymphocytes, hepatocytes, and fibroblasts.

For application of cell vehicles in the context of disseminated diseases, a cellular vector should possess the attributes of systemic distribution and appropriate tropism, and should be readily available. In this regard, circulating endothelial progenitors have recently been described [166, 167]. Phenotypically, these cells are characterised by the expression of the cellular surface markers CD34 and Flk-1, a receptor for vascular endothelial growth factor. A very intriguing aspect of their behaviour, originally described in animal models of limb ischaemia, is their capacity to localise into areas of angiogenesis after their systemic administration. A variety of genes could conceivably be introduced in these cells, and expression of genetic payloads could be obtained in the environment where these cells ultimately localise. A locoregional effect subsequent to the expression of the therapeutic gene would thus be achieved in areas otherwise poorly accessible to gene transfer. Therefore, endothelial progenitors may represent a novel cellular vector approach with unique features, based on their capacity for systemic circulation and their peculiarly advantageous natural tropism to areas of active angiogenesis. To be exploitable in a gene therapy context, however, it is critical for these endothelial progenitors to be primarily amenable to efficient and safe genetic modification for delivery of the payload therapeutic genes. Unfortunately, genetic modification of human and non-human primate $CD34^+$ cells with a variety of viral vectors has been persistently hampered by very low efficiency. Efforts are currently undergoing in several laboratories, including ours, to improve gene transfer into $CD34^+$ cells without unduly compromising their phenotype and function by using novel vectors *ex vivo*, and for evaluating the potential of endothelial progenitors for systemic gene delivery into metastatic cancer.

In addition to autologous cells, gene therapy based on genetic modification of non-autologous cells has been attempted. Protection within immuno-isolating devices would allow implantation of well-established recombinant cell lines in different hosts, offering a cost-effective approach to gene therapy of cancer when long-term treatment is required [168].

Genetic immunopotentiation to break immune tolerance to tumours

Cancer immunotherapy is yet to be realised as a therapeutic approach in the oncologist's armamentarium. New

ways to consider the immune response against tumours are probably needed if gene transfer is going to be applied in a clinically relevant way. Novel gene therapy approaches that exploit the accumulating knowledge on cytokines and cells involved in the immune response are mounting. They have been reviewed extensively [169, 170]. We would rather first emphasise a novel conceptual framework developed in recent years that can offer new insights on the entire approach of cancer immunotherapy. Secondly, we will focus on gene therapy strategies that, within this theoretical framework, seem particularly apt for offering useful biological information and therapeutic potential.

Danger versus tolerance. The classical paradigm of tumour immunology considers the responses of the immune system to follow a model of discrimination between 'self' and 'non-self' antigens. According to this paradigm, cancers, as microbes, are 'non self' and a major function of the immune system is to seek out and destroy new cancers as they arise. The practical corollary has been a very intense effort to develop tumour 'vaccines'. However, an alternative theoretical model has been proposed by Polly Matzinger to explain and modulate the relationship between the immune system and a genetic disease [171]. In this view, termed the 'danger' model, the need to defend the organism against exogenous lethal pathogens and the need to avoid lethal auto-immunity are equally balanced. According to this new paradigm, to avoid auto-immunity the default reaction of T cells to antigens on non-haematopoietic tissues is tolerance, and it is the role of the antigen-presenting cells to detect and report to T cells situations of dangerous tissue distress (for instance, the beginning of either an inflammatory reaction or tissue damage) that are worth its activation into cytotoxic T cells [84, 172]. If tissue cells normally induce tolerance in susceptible T cells, it is predicted that the default immune response to tumour antigens occurring in those tissues is tolerance as well.

This model can change the emphasis applied in certain immunotherapy strategies. In the classical model, importance is given to the identification of tumour antigens and elaboration of vaccines based on these antigens. Furthermore, it is expected that once activated, the immune response against cells bearing tumour antigens will proceed until their complete elimination. In contrast, the danger model would suggest potentially more relevant new goals such as the orchestration of inflammatory processes in tumour foci, the activation of dendritic cells and other antigen presenting cells, and the direction of T lymphocytes towards the tumour. In other words, the aim should be to recruit not only the adaptive immune response but also and most importantly the cells (macrophages, neutrophils, NK cells) and mediators (cytokines, chemokines) of the innate immune system [84] that establish the immune response in the context of activating 'danger', and make it distinct from tolerogenic immune responses. Importantly, these manoeuvers, including vaccination, should be maintained until elimination of the tumour to avoid its default tolerogenic effects.

Polynucleotide immunisation. Pursuant to the successful application of the strategies of mutation compensation and molecular chemotherapy, obtaining vector targeting and amplification is a critical goal. In contrast, for some genetic immunopotentiation strategies, it may appear that a sophisticated vector is not absolutely needed to facilitate the otherwise inefficient transfer of DNA into tumour or immune system cells.

The possibility exists for eliciting potent, prolonged, and specific immune responses through the intramuscular injection of fragments of nucleic acid encoding tumour-associated antigens [173, 174]. This so-called 'polynucleotide immunisation' (PNI) approach offers several advantages with respect to classic protein immunisation. First, synthesis of the antigen (or antigens) in eukaryotic cells *in vivo* is more likely to result in a protein that is correctly folded and with its antigenic domains adequately presented. Second, PNI elicits a $CD8^+$ cytotoxic T lymphocyte response in addition to a humoral response. Third, long term expression of the encoded antigen may favour long-lived immunity. Of note, the danger model would recommend that, to avoid toleration, repetitive immunisations that involve local inflammatory responses should be administered to keep the association between danger signals and the encoded antigens. Fourth, several nucleotides could easily be combined for induction of responses against multiple relevant antigens. Fifth, safety concerns related to virus-derived or cell derived vaccines are obviated. Sixth, manufacturing and use of recombinant DNA may have economical and logistic advantages with respect to standard vaccines. Polynucleotides in the form of both DNA and RNA can be used. For example, plasmid DNA encoding carcinoembryonic antigen, a non-transforming tumour-associated antigen, has shown prolonged humoral and lympho-proliferative responses in non-human primates [174], and is being tested in a clinical protocol for colorectal cancer patients. Transforming tumour-associated antigens, such as erbB-2, may be encoded by RNA constructs that avoid the risk of integration of a potentially oncogenic sequence and are expressed only transiently. Once the antigen is expressed in myofibres, its presentation to the effector cells follows an unknown pathway, but is known to induce antibody production, T cell proliferation, lymphokine release, generation of CTL, and delayed hypersensitivity reactions. Importantly, encouraging results in animal models have been followed by clinical trials for both immune protection and therapeutic applications. Although tumours are antigenically heterogeneous, the hypothesis is that immune responses against the polynucleotide-encoded antigens can break immune tolerance for the tumour via a single epitope, which, in turn, would alert the immune system to the existence of the tumour as a foreign entity, provoking a systemic response.

Enhanced antigen presentation by genetically modified dendritic cells. As we reviewed above, most tumours are ignored by the immune system. Thus, tumour antigen-specific T lymphocytes, which are certainly present in the immune repertoire, are not activated and migrate systemically without showing any special tropism towards its cognate antigens present in the tumour sites. This has been partly attributed to a lack of activation and antigen presentation by dendritic cells (DCs) in tumours [175, 176]. Indeed, DCs infiltrating several tumours lack B7-1 and B7-2 molecules, which reveals a non-stimulatory status and impedes the encounter by T lymphocytes of the required 'signal 2' on DCs for antigen-specific activation. However, when autologous DCs are expanded and exposed *ex vivo* to tumour antigens and these DCs are then reinfused, activation of tumour-specific cytotoxic T lymphocytes ensues. In animal models, this intervention achieves a protective effect against subsequent exposure to tumours and also can induce a therapeutic effect in tumours already present [175]. This strategy is currently being explored in patients [177].

Multiple vectors are being tested for delivering tumour antigens into DCs, including viral vectors, naked DNA, RNA, tumour lysates, and peptides [178, 179]. It is possible that methods that maximise exposure of DCs to a variety of tumour antigens may have an advantage by overcoming the expected emergence of antigen-loss variants as well as natural immunovariation of tumours [180]. Importantly, fusion of DCs and tumour cells have also shown the capacity to revert established immune tolerance [181]. This concept has been tested in transgenic animals tolerant to the antigen MUC1, and refractory to vaccination with irradiated MUC-1 positive cells. Immunisation with the dendritic cell fusion that express MUC1 resulted in the rejection of established metastases and there was no apparent autoimmunity against normal tissues. These findings demonstrate that tolerance to tumour-associated antigens can be reversed, and suggest that immunisation with hybrids of dendritic and carcinoma cells may be a powerful methodology for whole cell vaccination against cancer.

Reversal of tumour-induced immunosuppression. DCs are conceived as a powerful way to stimulate tumour-specific T cells. It may be, however, that a robust generation of such cytotoxic T cells is not enough for rejecting established tumours, as has been shown in elegant animal models [182]. Indeed, this can be predicted from the anatomy of the T cell response, whereby it is critical not only for DCs to uptake tumour antigens in the tumour site, mature and migrate to lymph nodes, and present antigens to T lymphocytes. But it is additionally needed for stimulated antigen-specific T lymphocytes to home assertively into the possibly widespread tumour sites, and keep their activation and proliferation therein, despite numerous immunosuppressive signals. Further strategies may be needed, therefore, for (1) attracting and activating the tumour-specific T cells into various tumour sites [183, 184]; (2) inhibiting the local production of immunosuppressive molecules, such as TGF beta [185], interleukin-10, VEGF, and fas ligand; and (3) counteracting the antigen variation and down-regulation of antigen presentation. It can be concluded that a systematic intervention with multiple targets at the different pathophysiological levels mentioned seems a reasonable programme to achieve a meaningful antitumour immune response.

CONCLUSION

The delineation of the molecular basis of cancer allows the possibility of specific intervention at the molecular level for therapeutic purposes. To this end, three main approaches have been developed: mutation compensation, molecular chemotherapy, and genetic immunopotentiation. For each of these conceptual approaches, human clinical protocols have entered testing in phases I and II to assess dose escalation, safety and toxicity issues, and more recently to evaluate efficacy, respectively. However, major problems remain to be solved before these approaches can become effective and common place strategies for cancer. Principle among these is the basic ability to deliver therapeutic genes quantitatively, and specifically, not only into tumour cells but also into tumour-supporting tissues and effector cells of the immune system. As vector technology fulfils these stringent requirements, it is anticipated that the promising results already observed in pre-clinical studies will translate quickly into the clinic for amelioration of life-threatening malignant diseases.

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Viewpoint: Are Studies in Genetically Altered Mice Out of Control?

Curt D. Sigmund

Abstract—Because the use of transgenic and gene-targeted models has increased in popularity, the number of reports describing unpredictable phenotypic effects caused by variation in the genetic background used to generate or propagate these models has steadily increased. There are now many examples in which animals containing the same exact genetic manipulation exhibit profoundly different phenotypes when present on diverse genetic backgrounds, demonstrating that genes unrelated, per se, to the ones being targeted can play a significant role in the observed phenotype. Herein, I will discuss (1) the source of genetic variability in mutant mouse models, (2) the appropriateness of using inbred mice as controls, and (3) strategies to help minimize genetic variation between experimental and control mice. (*Arterioscler Thromb Vasc Biol.* 2000;20:1425-1429.)

Key Words: transgenic mice ■ knockout mice ■ genetics ■ epigenetic

It is well documented that many physiological parameters in mammals are genetically determined. Therefore, it should not come as a surprise that many of the phenotypes examined in transgenic and knockout models are influenced by the genetic background in which they are studied. Genetic background is the collection of all genes present in an organism that influences a trait or traits. These genes may be part of the same biochemical or signaling pathway or of an opposing pathway or may appear unrelated to the gene being studied. Although all mouse strains contain the same collection of genes, it is allelic variation (sequence differences) and the interactions between allelic variants that influence a particular phenotype. These "epigenetic" effects can dramatically alter the observed phenotype and therefore can influence or alter the conclusions drawn from experiments.

Studies performed over the past few years have clearly illustrated that phenotypes caused by specific genetic modifications are strongly influenced by genes unlinked to the target locus. For example, whereas deletion of the p53 tumor suppressor gene causes a dramatic increase in the frequency of tumor formation in those mice compared with wild-type mice, the types of tumors formed, their numbers per animal, and age of tumor onset vary in different genetic backgrounds.[1–3] Other phenotypes observed in transgenic and gene-targeted animals influenced by genetic background include ethanol tolerance, sepsis, immunity, locomotor activity, behavior, organ structure, development, and cardiovascular physiology (Table). As examples of the latter, the incidence of stroke in mice deficient in tissue plasminogen activator and susceptibility to atherosclerosis in apoE-deficient mice differ when the knockout loci are present on C57BL/6, 129/Sv, or FVB/N backgrounds.[8,13]

On a positive note, phenotypic differences caused by allelic variation outside the target locus can provide a molecular genetic tool to identify and clone "modifier genes," which influence a phenotype.[17] However, as stated above, these differences can cause significant problems when interpreting and comparing the results of transgenic and knockout studies between laboratories.

The Problem

At the heart of the problem is genetic heterogeneity among strains used to generate transgenic and knockout mice. It is generally acknowledged that it is easier and more efficient to generate transgenic mice by using hybrid strains derived from 2 different genetic backgrounds. Presumably, this is because hybrid strains exhibit superior reproductive performance, are easier to superovulate, and have higher quality embryos for microinjection, a phenomenon referred to as "hybrid vigor."[18,19] Because of this, many transgenic laboratories use embryos derived from F_2 crosses of C57BL/6×SJL (B6SJL) or C57BL/6×DBA/2 (B6D2), among other combinations. When hybrid strains are used, each transgenic founder is genetically different from every other founder. This leaves the investigator with a choice of either continuing to breed their transgenic mice with hybrid strains to propagate the lines or, in cases in which genetic background issues are recognized and likely to be important, to generate congenic strains (defined below) by successive backcross breeding to 1 inbred strain, typically C57BL/6. Less frequently used are

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Phenotypes Exhibiting Genetic Background Effects in Knockout and Transgenic Mice

Phenotype	Genetic Alteration	Mouse Strains Tested	References
Tumor incidence	T antigen transgenic	C57BL/6, C57BL/6×NZW	4
	p53 knockout	129/Sv, C57BL/6×129/Sv	1, 2
	p53 knockout	VM, C57BL/6×129/Sv	3
	WAP-ras transgenic	FVB/N, SJL, C57BL/6, CD1	5
	Tsc2 knockout	Black Swiss, 129/SvJae	6
Ethanol tolerance	PKCγ knockout	C57BL/6×129/Sv, C57BL/6	7
Atherosclerosis	ApoE knockout	FVB/N, C57BL/6	8
Sepsis	IL-4 knockout	129/Sv, C57BL/6	9
Locomotor activity	D2R knockout	129/SvEv×C57/BL6	10
		129/SvEv, C57BL/6	
	βAPP knockout	129/Sv, C57BL/6	11
		129Sv×C57BL/6	
Forebrain structure	βAPP knockout	C57BL/6, 129/SvEv	12
Stroke	tPA knockout	C57BL/6×129/Sv, C57BL/6	13
Renal development	AT1AR knockout	129/Sv, C57BL/6, 129Sv×C57BL/6	14
	AGT knockout	C57BL/6×CBA, C57BL/6	15
Development and pleiotropic effects	EGFR knockout	129/Sv, CD-1	16

PKC indicates protein kinase C; IL, interleukin; tPA, tissue plasminogen activator; and EGFR, epidermal growth factor receptor.

transgenic mice generated directly on inbred strains. The inbred FVB/N strain is used by some laboratories because it exhibits excellent reproductive performance, it has large litters, and the 1-cell fertilized embryos have prominent and easily injectable pronuclei.[19] One limitation is that it is genetically distinct from the C57BL/6 strain, which is used by many investigators.

A second issue specifically related to transgenic mice (but not gene-targeted mice) is the position effect. Because of the random nature of the transgene insertion event after pronuclear injection, each resultant founder contains the transgene at a different site in the genome. These position effects can profoundly influence transgene expression and, therefore, the observed phenotype.[20,21] This occurs because transcriptional regulatory elements present at or near the site of insertion (controlling the expression of a nearby gene or gene cluster) could impart new instructions on the transgene. Consequently, it is essential that several independent lines of mice, derived from founders with different insertion sites, are examined before a conclusion relating a phenotype to a specific pattern of transgene expression is made.

When performing gene targeting in embryonic stem (ES) cells, position effects are essentially eliminated but not the effects caused by genetic variability. As in the transgenic experiments, this results from the generation of hybrid strains. Most commonly used ES cell lines are derived from strain 129, and a number of 129 substrains are in existence (129/Sv, 129/SvEv, and 129/Ola), further complicating the scenario. As mentioned above, hybrid vigor has been reported for the viability of ES cell lines.[22] Although many (but not all) ES cell lines are themselves inbred, most investigators report that the 129 strain exhibits poorer reproductive performance than other inbred strains and also exhibits other abnormalities, including development of teratocarcinoma. This has prompted most investigators to breed their chimeras to C57BL/6, thus generating a hybrid mouse that is heterozygous (+/−) at the target locus and an F_1 between C57BL/6×129 at all other loci. The F_1 mice are all genetically identical because they inherit 1 chromosomal complement each from the 129 and C57BL/6 strains. However, when they are intercrossed to generate a mouse homozygous (−/−) for the target locus, the resultant offspring become an F_2 of the parent strains. Therefore, whether wild-type, heterozygous, or homozygous for the target locus, the offspring have a random mix of 129 or C57BL/6 chromosomal DNA throughout the genome. The maintenance of a strain homozygous at the target locus by continuous inbreeding of these F_2 mice can eventually select for phenotypic changes because loci causing deleterious effects are lost, and those providing a survival advantage are retained. Consequently, maintenance of the targeted locus in this manner is not recommended. Therefore, investigators are again left with the option to retain the mixed genetic background of the strains or to generate congenic strains.

Further complicating this problem has been the marked increase in the generation of double-knockout strains and the combinatorial use of knockout and transgenic rescue. In the latter, transgenes expressed either systemically or tissue-specifically are transferred into a knockout mouse to rescue some altered phenotype (often lethality).[23] Moreover, the development of inducible transgenes and methods using the cre-loxP recombinase system to generate cell-specific knockouts will necessitate the introduction of multiple transgenes into a single genetic background. Clearly, it will become important to avoid the creation of a mixed genetic background so complex as to preclude any reasonable use of controls and prevent replication by other investigators.

Effective Experimental Strategies

Clearly, when transgenic and gene-targeting experiments are designed, the ideal situation would have control mice that are

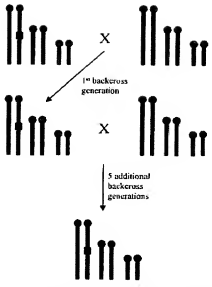

Figure 1. Generation of a congenic strain. A schematic representation of the chromosomal content in the generation of a congenic strain is shown. The 129 chromosomes are red, and the C57BL/6 chromosomes are blue. A targeted modification induced in ES cells is shown as a solid block box. Only 3 chromosomes are shown for simplicity. The top left illustrates a heterozygous knockout after germ-line transmission through a chimera. This mouse is heterozygous at the target locus, with an F_1 between 129 and C57BL/6 at all loci. To generate the congenic strain, the F_1 is successively backcrossed to a C57BL/6 mouse (all blue), and the targeted locus is selected in all offspring. The 129 genome is progressively diluted in each backcross because of the random assortment of chromosomes and homologous recombination. After 6 generations of backcross breeding, the resultant offspring are >99% C57BL/6 except for the region surrounding the targeted modification, which remains derived from the 129 strain.

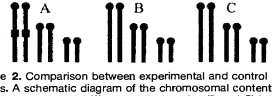

Figure 2. Comparison between experimental and control strains. A schematic diagram of the chromosomal content between experimental (A) and control strains (B and C) is shown. The experimental mouse is homozygous for the targeted disruption (solid black box) and is a C57BL/6 (blue) congenic strain. A small amount of 129 genomic DNA (red) upstream and downstream from the target locus remains. A C57BL/6 inbred mouse (B) is blue at all loci, and although it lacks the targeted modification, it also lacks the 129 DNA linked to the disruption. The ideal control (C) would be a C57BL/6 congenic strain with a similar extent of 129 DNA as shown in panel A.

genetically identical to the experimental mice. The use of isogenic strains differing only in the presence or absence of the target locus would be the "gold standard." However, this can only be achieved if inbred mice are used for the generation of experimental models. Therefore, whenever possible, inbred strains should be used as the choice of controls becomes obvious. As discussed above, when this is not a practical or feasible option, the next best alternative is to develop a program of continuous inbreeding to a common strain, thus generating congenic mice. A congenic strain is one that is genetically identical to a control strain except for a single region of 1 chromosome (Figure 1). In the context of this discussion, this refers either to the target locus or the inserted transgene. The generation of congenic strains also provides an opportunity to place the target locus on a number of different genetic backgrounds and thus directly test for strain-specific modifier loci.

Although the generation of congenic mice is simple, requiring only accurate record keeping, it can be time-consuming and expensive, especially when multiple lines must be developed. Six generations of backcross breeding (2 years) is required before the genetic backgrounds are statistically >99% homogeneous, and the return on additional generations of backcross breeding markedly diminishes thereafter. For example, it requires 4 additional generations to increase genetic homogeneity from 99.2% to 99.95%. The use of a speed-congenic approach or a combination of in vitro fertilization and prepubertal superovulation can be used to decrease the time needed to generate congenic strains.[24,25] The speed-congenic approach makes use of the well-developed genetic map of the mouse, thus affording an opportunity to screen the DNA of each offspring generated along the route toward congenic production to select for mice containing markers from the appropriate genetic background at the target locus and elsewhere.[26,27] Those mice that are "further" along in congenic development than expected, on the basis of random segregation alone, can be selected by this process for further breeding.

After a C57BL/6 congenic knockout strain is derived, either nontransgenic littermates or age-matched inbred C57BL/6 mice should serve as reasonable controls. However, it is important for the researcher to appreciate that even this scenario has weaknesses. Indeed, 1 limitation of using littermate or wild-type mice as controls for congenic transgenic (or knockout) strains is that some parental genomic DNA upstream and downstream from the target locus (129 DNA in the case of ES cell–derived gene targeting) remains. By use of the same example as described above, this occurs because as the targeted modification (made in 129 genomic DNA) is introgressed into the C57BL/6 strain, the targeted locus and therefore 129 DNA in the vicinity of (or linked to) the locus will be selected in each backcross generation (Figure 2A). The location of the breakpoint between C57BL/6 and 129 genomic DNA upstream and downstream from the target locus will depend on where the recombination between the 2 genomes occurred. Therefore, nontransgenic littermates and wild-type C57BL/6 mice will lack the targeted locus and also the closely linked 129 genomic DNA (Figure 2B). In some cases, this DNA may contain closely linked modifier genes. This may be especially critical when examining large gene families, which may have members closely clustered in the genome.

Therefore, the optimal control would be a congenic control strain containing a similar amount of foreign genomic information around the target locus but lacking the targeted modification itself (Figure 2C). Absolutely identical congenic strains cannot be generated. However, similarities between control and experimental strains can be maximized by taking advantage of the dense genetic map of the mouse and the thousands of polymorphic microsatellite markers distributed throughout the mouse genome.[28] For example, control mice can be selected that contain C57BL/6-specific markers throughout the genome except in the region of the target

locus, where 129-specific markers would be selected. These mice can then be propagated to generate a control congenic strain that is similar to the experimental mouse but lacks the targeted ES cell–induced modification.

Of course, we must recognize that from a practical standpoint, circumstances will often dictate assessing the phenotype of a knockout mouse long before a congenic strain can be generated. If these mice were derived from 129 ES cells and the chimera was bred to C57BL/6, it is predictable that each mouse, while containing the same targeted modification of the genome, will be genetically different at all other loci because of random segregation and recombination in the F_2 generation. In this case, it would be inappropriate to use either inbred C57BL/6 or 129 mice solely as controls. Instead, wild-type or heterozygous littermates from the same breedings should be included as well. The use of littermates would help minimize environmental variability in such experiments. Moreover, larger numbers of mice should be examined to ensure that the range of phenotypes possible due to epigenetic interactions with the genetic background is observed. Although not genetically identical, when examined as a population, the experimental and control groups could be considered "genetically similar." Once the phenotypes are assessed, it would be prudent to generate congenic strains and reexamine the phenotype in the resultant animals.

Guidelines

Until such time as a standardized mouse strain exists that facilitates easy generation of transgenic and knockout mice, the debate over the proper use of experimental and control mice will continue. There are no easy solutions to this problem. As illustrated above, in the absence of inbred strains, there is no optimal set of experimental and control conditions that normalizes the epigenetic effects of unlinked loci. Therefore, it becomes the responsibility of the investigator to use common sense and design the best possible control experiments that fit the individual situation, to assess whether the phenotype observed in their model is due specifically to the targeted modification or is affected by other loci, and to inform the scientific community if phenotypic alterations become evident. The geneticists at the Banbury Conference on Genetic Background in Mice[29] in 1996 established 3 general guiding principals for the use of transgenic and gene targeted mice in neuroscience. These principals should be applicable to all disciplines, and the reader is referred to that article for a detailed discussion of options for designing such experiments.[29] Their guidelines state the following: (1) Published reports must include a detailed description of the genetic background of the mice studied that is sufficient enough to allow replication of the study. (2) The genetic background chosen for the studies should not be so complex as to preclude replication. (3) Use of common or standardized genetic background would facilitate comparison of experimental results among laboratories.

Minimally, these guidelines provide a common-sense approach that provides the reader with sufficient information to understand and potentially replicate reported results and also provide a framework to identify the causes of phenotypic variation observed in different laboratories. It makes common sense to recommend that these guidelines be adopted by all researchers using genetically modified mice as models of cardiovascular disease until such time as standardized strains are used universally.

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The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

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Paper No. 37

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte GARY L. CLAYMAN

MAILED
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PAT. & T.M. OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

Appeal No. 2000-0742
Application 08/758,033[1]

HEARD November 8, 2001

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PAT. & T.M. OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

Before WINTERS, MILLS, and GRIMES, Administrative Patent Judges.

GRIMES, Administrative Patent Judge.

REMAND TO THE EXAMINER

This appeal concerns claims 1-14, 16-20, 26-32, 36, and 37, which the examiner has rejected under 35 U.S.C. § 103 as being obvious over certain prior art references. We conclude that the examiner's rejection does not rely on the most relevant prior art in the record. Therefore, we vacate the rejection on appeal and remand the application to the examiner.

[1] Application for patent filed November 27, 1996. The instant application claims the benefit of priority under 35 U.S.C. § 119(e)(1) based on provisional application 60/007,810, filed November 30, 1995.

Claim 1 is representative of the subject matter on appeal and reads as follows:

1. A method of inhibiting growth of a p53-positive tumor cell in a mammalian subject with a solid tumor comprising the steps of:

(a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and

(b) directly administering said viral expression construct to said tumor in vivo, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth,

wherein said tumor comprises cells that express a functional p53 polypeptide.

The examiner relies on the following references:

Bramwell, "The role of chemotherapy in multimodality therapy," Canadian Journal of Surgery, Vol. 31, No. 5, pp. 390-396 (1988)

Cajot et al. (Cajot), "Growth suppression mediated by transfection of p53 in Hut292DM human lung cancer cells expressing endogenous wild-type p53 protein," Cancer Research, Vol. 52, No. 24, pp. 6956-6960 (1992)

Liu et al. (Liu), "Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus," Cancer Research, Vol. 54, pp. 3662-3667 (1994)

Wills et al. (Wills), "Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer," Human Gene Therapy, Vol. 5, pp. 1079-1088 (1994)

Zhang et al. (Zhang), "Gene therapy strategies for cancer," Exp. Opin. Invest. Drugs, Vol. 4, No. 6, pp. 487-514 (1995)

In the Final Rejection, the examiner also relied on the following references, which were withdrawn in response to a 131 declaration (Paper No. 25, filed November 9, 1999):

Katayose et al. (Katayose), "Cytotoxic effects of adenovirus-mediated wild-type p53 protein expression in normal and tumor mammary epithelial cells," Clinical Cancer Research, Vol. 1, pp. 889-897 (August 1995).

Srivastava et al. (Srivastava), "Recombinant adenovirus vector expressing wild-type p53 is a potent inhibitor of prostate cancer cell proliferation," Urology, Vol. 46, No. 6, pp. 843-848 (1995).

Claims 1-14, 16-20, 26-32, 36, and 37 stand rejected under 35 U.S.C. § 103 as obvious over the combined disclosures of Cajot, either of Wills or Liu, and either of Zhang or Bramwell.

We vacate and remand.

Background

1. Technical Background

Appellant's specification discloses a method of treating squamous cell carcinoma by administering an expression construct encoding the tumor suppressor p53. See page 3. The specification states that the "endogenous p53 of the squamous cell carcinoma may or may not be mutated." Id. The claims on appeal are directed to a disclosed embodiment that comprises treating cancer cells in which the endogenous p53 is not mutated, i.e., treating $p53^{+}$ tumor cells by administration of a construct which expresses p53.

> Regarding this embodiment, the specification states that
>
> it has now been observed that p53 gene therapy of cancers may be effective regardless of the p53 status of the tumor cell. Surpisingly, therapeutic effects have been observed when a viral vector carrying the wild-type p53 gene is used to treat a tumor, the cells of which express a functional p53 molecule. This result would not have been predicted based on the current understanding of how tumor suppressors function. It also is surprising given that normal cells, which also express a functional p53 molecule, are apparently unaffected by expression of high levels of p53 from a viral construct.

Id., page 7.

2. Procedural Background

In the examiner's Final Office Action, all of the claims on appeal were rejected under 35 U.S.C. § 103 as obvious over the combination of Cajot, either of Katayose or Srivastava, either of Wills or Liu, and either of Zhang or Bramwell. See Paper No. 17, mailed April 12, 1999, page 7.

In response, Appellant filed a declaration under 37 CFR § 1.131. See Paper No. 25, filed November 9, 1999. In his declaration, Appellant stated that he had published research papers in January 1995 and July 1995 that "reported the Ad-p53 infection of cell lines with both mutated and wild-type p53."[2] Paragraph 3. He also stated that he understood "that the Examiner in charge of examining the referenced application has

[2] The papers relied on are Liu et al., "Apoptosis induction mediated by wild-type p53 adenoviral gene transfer in squamous cell carcinoma of the head and neck," Cancer Research, Vol. 55, pp. 3117-3122 (July 15, 1995) and Clayman et al., "In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous cell carcinoma," Cancer Research, Vol. 55, pp. 1-6 (January 1, 1995).

previously taken the position that these papers teach the use of adeno-p53 in the therapy of tumors in vivo, including the therapy of p53-positive tumor cells." Id.[3]

Appellant also stated in his declaration that the 1995 research papers "demonstrate that [he] had achieved the subject matter they disclose in the United States at least as of their date of publication, the earliest publication date as between the two being January, 1995." Paragraph 4. He also noted that Katayose and Srivastava were published after January 1995 and concluded that "[b]ased on the earlier publication of [the] two articles referenced above, it is clear that [he] had in [his] possession at least equivalent, and indeed more extensive, data than is taught in the Katayose and Srivastava references at a time prior to their respective publication dates." Paragraph 5.

In response to the 131 declaration, the examiner withdrew her reliance on Katayose and Srivastava. See the Examiner's Answer, page 20.

Discussion

1. The rejection on appeal.

The claims stand rejected as obvious over the combined disclosures of Cajot, either of Wills or Liu, and either of Zhang or Bramwell. Cajot teaches transfection of human lung cancer cells with a plasmid vector expressing p53. The tumor cells

[3] Appellant cites an "Office Action of 2/17/99" as supporting his position. The file record, however, shows no Office Action that was mailed February 17, 1999, although one was mailed February 17, 1998. We

(Hut292DM) used by Cajot express endogenous p53. Cajot found that "growth suppression [was] induced by high level expression of exogenous wild-type p53 in lung cancer cells expressing normal endogenous p53 protein." Page 6956, right hand column. Cajot concluded that his "work extends the scope of the potential effectiveness of wild-type p53 to control tumor growth to recipient cells that contain no apparent defect in endogenous wild-type p53 expression." Page 6959, right-hand column.

As the examiner notes, however, Cajot does not teach "in vivo transduction with a viral expression construct encoding p53." Examiner's Answer, page 11. The examiner relies on the secondary references to remedy this deficiency. Liu and Wills both teach inhibiting the growth of $p53^-$ tumor cells in vivo by administering recombinant adenovirus constructs encoding p53. See Liu, page 3662, abstract ("In vivo studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-p53."); Wills, page 1079, abstract ("Continued treatment of H69 tumors with MLP/p53 recombinant led to reduced tumor growth and increased survival time.").[4]

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understand this 1998 Office action to be the one referred to by Appellant.

[4] Zhang and Bramwell are relied on for teaching the treatment of cancer by a combination of two or more therapeutic approaches (gene therapy, radiation, chemotherapy, etc.). These references are relevant to some of the dependent claims on appeal but are not required for the prima facie case with respect to representative claim 1.

The examiner concluded that it would have been obvious, in view of the combined teachings of these references, to "treat a tumor which comprises cells that express a functional p53 polypeptide with adenoviral vectors encoding p53 polypeptide with a reasonable expectation of success given that Cajot et al. specifically teaches that both p53 positive and p53 negative tumors can be inhibited by expression of exogenous p53." Examiner's Answer, page 13.

Appellant argues that the prior art does not support a prima facie conclusion of obviousness. Appellant argues that, if the claimed method was considered in the context of the prior art as a whole, a person of ordinary skill in the art would not have had a reasonable expectation that the claimed method would be successful. See the Appeal Brief, pages 17 and 18-19. Appellant also argues that the experiments disclosed by Cajot suffer from serious scientific flaws, "reducing its probative value to a nullity." Appeal Brief, pages 13 and 19-21. Appellant also argues that Katayose and Srivastava "evidence confusion in the field," and in any case are not prior art because they were removed by Appellant's 131 declaration. Appeal Brief, pages 13-18. Finally, Appellant argues that he has presented evidence of unexpected results, which overcome any prima facie case that might be established by the cited references. Appeal Brief, pages 21-23.

"The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be

carried out and would have a reasonable likelihood of success, viewed in the light of the prior art. Both the suggestion and expectation of success must be founded in the prior art, not in the applicant's disclosure." In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988) (citations omitted).

Obviousness under 35 U.S.C. § 103 is established if a preponderance of the evidence in the record supports the obviousness of the claimed invention. See In re Oetiker, 977 F.2d 1443, 1446, 24 USPQ2d 1443,1445 (Fed. Cir. 1992) ("[T]he conclusion of obviousness vel non is based on the preponderance of evidence and argument in the record."). In this case, both Appellant and the examiner have substantial evidentiary support for their positions; it would be a close question which has the weight of the evidence on their side. We need not weigh the evidence on each side so closely, however, because we conclude that the examiner has not relied on the most relevant prior art references in the record.

2. Rule 131

In the Final Rejection, the examiner relied on Katayose and Srivastava, in the alternative, in addition to the references relied on in the Examiner's Answer. As discussed above, Appellant filed a 131 declaration that pointed to two scientific papers that were published before Katayose or Srivastava, averred that the examiner treated these earlier papers as anticipatory references, and stated that Appellant was in possession of more data than Katayose or Srivastava, prior to the publication of

Katayose or Srivastava. See pages 4-5, supra. The examiner withdrew her reliance on Katayose and Srivastava in response to the 131 declaration.

When faced with a rejection based on a reference that is prior art under 35 U.S.C. §§ 102(a) or 102(e), a patent applicant may attempt to remove the reference as prior art by filing a declaration under 37 CFR § 1.131. "The purpose of filing a 131 declaration is to demonstrate that the applicant's date of invention is prior to the effective date of the reference cited in support of a rejection." In re Asahi/America Inc., 68 F.3d 442, 445, 37 USPQ2d 1204, 1206 (Fed. Cir. 1995). Thus, an effective Rule 131 declaration must show either "reduction to practice prior to the effective date of the reference, or conception of the invention prior to the effective date of the reference coupled with due diligence from prior to said date to a subsequent reduction to practice." 37 CFR § 1.131(b). It is the applicant's burden to decipher Rule 131 declaration evidence and explain its content. See In re Borkowski, 505 F.2d 713, 718, 184 USPQ 29, 33 (CCPA 1974).

In this case, the declaration does not allege either "reduction to practice prior to the effective date of the reference[s], or conception of the invention prior to the effective date of the reference[s] coupled with due diligence," as required by Rule 131. The declaration therefore fails to satisfy the express terms of the rule.

Instead, the declaration purports to antedate Katayose and Srivastava by "demonstrat[ing] that [Appellant] had achieved the subject matter they disclose in the United States at least as of their date of publication." Paragraph 4. Appellant contends

that because he had in his "possession at least equivalent, and indeed more extensive, data than is taught in the Katayose and Srivastava references at a time prior to their respective publication dates," id., paragraph 5, he has removed the references as prior art.

Appellant's position is not entirely without support in the case law. See, e.g., In re Stempel, 241 F.2d 755, 759, 113 USPQ 77, 81 (CCPA 1957) ("[U]nder the law all the applicant can be required to show is priority with respect to so much of the claimed invention as the reference happens to show.") However, the Stempel standard has long since been limited to the situation where the reference being antedated showed a species within a later-claimed genus. See In re Tanczyn, 347 F.2d 830, 832, 146 USPQ 298, 300 (CCPA 1965). The Tanczyn court distinguished that situation from one in which the claims were rejected under § 103 over a combination of references. See id. at 832, 146 USPQ at 300-301 ("The mere fact that an applicant has previously produced that which is disclosed by a reference, however, may have no bearing on the problem of whether he made his invention or a patentable portion of it before the date of a reference."). The Tanczyn court concluded that

> [t]he primary consideration is whether, in addition to showing what the reference shows, the affidavit also establishes possession of either the whole invention claimed or something falling within the claim, in the sense that the claim as a whole reads on it.

> It is not sufficient to show in a Rule 131 affidavit that an invention wholly outside of that being claimed was made prior to the reference date. Such fact is irrelevant.

Id. at 833,146 USPQ at 301 (emphasis in original). See also Borkowski, 505 F.2d at 719, 184 USPQ at 33-34 (Rule 131 requires "a factual showing of completion of the invention before the critical date.").

Here, Appellant's 131 declaration does not assert that the research papers he relies on show "possession of either the whole invention claimed or something falling within the claim, in the sense that the claim as a whole reads on it," as required to antedate the Katayose and Srivastava references. See Tanczyn, 347 F.2d at 833, 146 USPQ at 301. Appellant asserts only that he had in his "possession at least equivalent, and indeed more extensive, data than is taught in the Katayose and Srivastava references at a time prior to their respective publication dates." 131 declaration, paragraph 5. The Tanczyn court, however, noted that "[t]he mere fact that an applicant has previously produced that which is disclosed by a reference . . . may have no bearing on the problem of whether he made his invention or a patentable portion of it before the date of a reference." Tanczyn, 347 F.2d at 832, 146 USPQ at 300-301.

It is Appellant's burden to decipher the declaration evidence and explain its content. See Borkowski, 505 F.2d at 718, 184 USPQ at 33. Appellant's 131 declaration makes no attempt to explain the evidence relied on, i.e., the data in the Liu

and Clayman papers, and the data in the published papers do not appear to show reduction to practice of the method now claimed.

The instant claims are directed to treatment of "a mammalian subject with a solid tumor." See claim 1. Both papers, by contrast, disclose experiments in which the growth of tumor cells injected into nude mice was prevented by adenoviral vectors expressing p53. See Clayman, page 1, abstract ("[W]e prevented the establishment of tumors in nude mice in which tumor cells had been s.c. implanted by transiently introducing exogenous wild-type p53 via an adenoviral vector 2 days following tumor cell implantation."); Liu, page 3117, abstract ("For in vivo analysis of apoptosis, nude mice in which squamous cell carcinoma of the head and neck cell lines had been implanted s.c. had exogenous wt-p53 transiently introduced to the tumor cells via Ad5CMV-p53 2 days later. In situ end labeling clearly illustrated apoptosis in the tumor cells."). Since the methods disclosed in the papers are directed to prevention of tumor cell growth, the papers do not show treatment of "a mammalian subject with a solid tumor."

Appellant's 131 declaration does not aver that the papers show reduction to practice of the instant claims. Nor does the declaration aver that the papers show conception of the method now claimed, plus diligence. All that the declaration avers is that Appellant had in his possession the same data disclosed by Katayose and Srivastava, before their respective dates of publication. This showing is facially

inadequate to antedate the references, and the examiner erred in withdrawing Katayose and Srivastava as prior art based on the 131 declaration.

3. Conclusion

The disclosures of Katayose and Srivastava are very relevant to the claimed method. Both Katayose and Srivastava disclose treatment of $p53^+$ tumor cells using adenoviral vectors which express wild-type p53, just as in the claimed method and in contrast to the experiments disclosed by Cajot, Baker, and Casey,[5] all of whom use a plasmid vector. Katayose and Srivastava both show positive results using the adenoviral vector. Thus, Katayose and Srivastava appear to be very relevant to the issue of the patentability of the instant claims under 35 U.S.C. § 103. Since Katayose and Srivastava are apparently available as prior art under 35 U.S.C. § 102(a), the evidence they disclose should be considered in the obviousness analysis.

The obviousness or nonobviousness of a claimed invention should be determined based on the most relevant prior art. See In re Gorman, 933 F.2d 982, 986, 18 USPQ2d 1885, 1888 (Fed. Cir. 1991); (The test of obviousness is "whether the teachings of the prior art, taken as a whole, would have made obvious the claimed invention."); In re Hedges, 783 F.2d 1038, 1041, 228 USPQ 685, 687 (Fed. Cir. 1986)

[5] Baker et al., "Suppression of human colorectal carcinoma cell growth by wild-type p53," Science, Vol. 249, pp. 912-915 (1990), and Casey et al., "Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene," Oncogene, Vol. 6, pp. 1791-1797 (1991), are cited by Appellant as evidence showing that those skilled in the art would not have had a reasonable expectation of success in practicing the claimed method.

(When determining obviousness, "the prior art as a whole must be considered. The teachings are to be viewed as they would have been viewed by one of ordinary skill."). Since we conclude that the rejection on appeal does not rely on the most relevant prior art in the record, we vacate that rejection and remand the application to the examiner.

Upon return of this application, the examiner should reconsider whether Appellant's declaration meets the requirements of 37 CFR § 1.131 with respect to the claimed invention and review the patentability of the claimed process based on the prior art as a whole. After doing so, she should reject the claims, if appropriate, based on the best available prior art.

Summary

Appellant's Rule 131 declaration is facially defective and should not have been found sufficient to antedate Katayose and Srivastava. Since the evidence provided by Katayose and Srivastava may be crucial to the patentability of the claims on appeal, we vacate the rejection and remand.

VACATED AND REMANDED

SHERMAN D. WINTERS)
Administrative Patent Judge)
)
)
DEMETRA J. MILLS) BOARD OF PATENT
Administrative Patent Judge) APPEALS AND
) INTERFERENCES
)
ERIC GRIMES)
Administrative Patent Judge)

Appeal No. 2000-0742
Application No. 08/758,033

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte GARY L. CLAYMAN

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BOARD OF PATENT APPEALS
AND INTERFERENCES

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By: Checked

Appeal No. 2000-0742
Application 08/758,033

ORDER REMANDING TO EXAMINER

An Amendment after Final was filed on April 19, 2000 (Paper No. 32, Amendment E). There is no indication on the record as to whether the examiner has considered this amendment and whether the entry of the amendment into the record was granted or denied.

A Reply Brief was filed April 19, 2000 (Paper No. 30). There is no indication on the record as to whether the examiner has considered this Reply Brief and whether the entry of the Reply Brief in to the record was granted or denied.

Accordingly, it is

ORDERED that the application be remanded to the examiner for consideration of the aforementioned amendment after final and the Reply Brief. A decision on this after final amendment and Reply Brief must include a written

notification to appellant and must include any such further action as may be appropriate.

It is important that the Board of Patent Appeals and Interferences be informed promptly of any action affecting the appeal (i.e., abandonment, issue, reopening prosecution).

BOARD OF PATENT APPEALS

AND INTERFERENCES

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